การศึกษาระบาดวิทยาเชิงโมเลกุลของเชื้อโบคาไวรัสและเชื้อโบคาไวรัส 2

นาย ทวีศักดิ์ เชี่ยวชาญศิลป์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

MOLECULAR EPIDEMIOLOGY OF HUMAN BOCAVIRUS AND NEW DISCOVERY HUMAN BOCAVIRUS 2

Mr. Thaweesak Chieochansin

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2008

Copyright of Chulalongkorn University

Thesis Title	MOLECULAR EPIDERMIOLOGY OF HUMAN BOCAVIRUS AND NEW DISCOVERY HUMAN BOCAVIRUS 2
By	Mr.Thaweesak Chieochansin
Field of Study	Biomedical Sciences
Advisor	Professor Yong Poovorawan, M.D.
Co-Advisor	Associate Professor Rujipat Samransamruajkit, M.D.

Accepted by the the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of Graduate School
(Associate Professor Pornpote Piumsomboon,Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor Kanisak Oraveerakul, D.V.M , Ph.D.)

..... . Advisor
(Professor Yong Poovorawan, M.D.)

..... .Co-Advisor
(Associate Professor Rujipat Samransamruajkit, M.D.)

..... Examiner
(Associate Professor Parvapan Bhattarakosol, Ph.D.)

..... External Examiner
(Associate Professor Chantapong Wasi, M.D.)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ทวีศักดิ์ เชี่ยวชาญศิลป์ : การศึกษาระบาดวิทยาเชิงโมเลกุลของเชื้อโบคาไวรัสและเชื้อโบคาไวรัส 2. (MOLECULAR EPIDEMIOLOGY OF HUMAN BOCAVIRUS AND NEW DISCOVERY HUMAN BOCAVIRUS 2) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.นพ.ยง ภู่วรวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.นพ.รุจิภักดิ์ สำราญสำรวจกิจ, 60หน้า.

การศึกษานี้ได้ทำการสำรวจเชื้อโบคาไวรัส และไวรัสที่มีการค้นพบใหม่คือ โบคาไวรัส 2 จากตัวอย่างสารคัดหลั่งทางเดินหายใจของผู้ป่วยเด็กที่มีอาการทางเดินหายใจอักเสบ และอุจจาระของผู้ป่วยเด็กที่มีอาการอุจจาระร่วง เปรียบเทียบกับเด็กที่ไม่มีอาการทางระบบทางเดินอาหาร พบว่าสามารถตรวจพอสารพันธุกรรมของเชื้อโบคาไวรัสในตัวอย่างสารคัดหลั่งสารทางเดินหายใจและจากอุจจาระในผู้ป่วยเด็กที่มีอาการท้องเสีย เท่ากับ 6.62% และ 0.9% ตามลำดับ ไม่พบในอุจาระของเด็กที่ไม่มีอาการทางระบบทางเดินอาหาร

ในการศึกษานี้ยังพบการติดเชื้อร่วมระหว่างเชื้อโบคาไวรัสกับเชื้อไวรัสทางระบบทางเดินหายใจอื่น ๆ เท่ากับ 40% และไม่พบโรคที่เฉพาะเจาะจงต่อการติดเชื้อโบคาไวรัส จากการวิเคราะห์รหัสพันธุกรรมและความสัมพันธ์เชิงวงศ์วานวิทยาพบว่าเชื้อโบคาไวรัสเป็นเชื้อที่มีความหลากหลายทางพันธุกรรมต่ำมาก ยีนที่พบความหลากหลายมากที่สุดคือ VP1 และ VP2

นอกจากนี้ยังได้ทำการศึกษาระบาดวิทยาของเชื้อโบคาไวรัส 2 ซึ่งเป็นเชื้อที่เพิ่งค้นพบและจำแนกได้ใหม่ในปี พ.ศ. 2552 ที่แสดงความใกล้เคียงกับเชื้อโบคาไวรัสที่เป็นสปีชีส์เดิมประมาณ 74% จากการศึกษาพบว่าสามารถตรวจพบเชื้อโบคาไวรัส 2 ได้เฉพาะในตัวอย่างอุจาระเด็กที่มีอาการทางระบบทางเดินอาหารทั้งในตัวอย่างจากประเทศไทย (0.61%) และตัวอย่างจากประเทศอังกฤษ (0.64%) พบว่าเชื้อโบคาไวรัส 2 แสดงความหลากหลายทางพันธุกรรมในระดับที่มากกว่าเชื้อโบคาไวรัส

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....
ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4789659120 : MAJOR BIOMEDICAL SCIENCE

KEYWORDS : HUMAN BOCAVIRUS / HBoV2 / RESPIRATORY INFECTION / GI TRACT INFECTION

THAWEESAK CHIEOCHANSIN : MOLECULAR EPIDEMIOLOGY OF HUMAN BOCAVIRUS AND NEW DISCOVERY HUMAN BOCAVIRUS2. ADVISOR : PROF. YONG POOVORAWAN, CO-ADVISOR: ASST. PROF. RUJIPAT SAMRANSAMRUAJKIT, 60 pp.

The recently discovery virus base on metagenomic technique, human bocavirus (HBoV), had been screening in this study. Nasopharyngeal (NP) aspirate and stool from acute diarrhea patients and healthy children were corrected. The result showed that HBoV can be detected in NP aspirate 6.62% and diarrhea stool samples 0.9%. Where as could not be detected in healthy children stool samples. The co-infection with other respiratory viruses also found (40%) and there were no clinical specific manifestations for HBoV; however, fever and productive cough were commonly found. The results from complete coding sequences showed that the most conserved regions of HBoV are the NS1 and NP1 genes, whereas VP1 and VP2 showed frequent variations.

The epidemiological study of newly discovered species of *Bocavirus* genus, HBoV2, which show about 74% amino acid identity when comparing with HBoV had been also focusing in this study. HBoV2 could be only found in diarrhea stool samples that collected in both Thai (0.61%) and UK patients (0.64%). Moreover, it has shown higher variation at the genome level than HBoV. In conclusion the molecular epidemiological study of HBoV and HBoV2 provided more information of these viruses.

Field of Study : Biomedical sciences Student's Signature : Thaweesak Chieochansin

Academic Year : 2008 Advisor's Signature : Yong Poovorawan

Co-Advisor's Signature : Sm

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my adivisor, Professor Yong Poovorawan, for his competent supervision, guidance, encouragement and criticism which have inspired me to accomplish my study.

I am extremely grateful to my thesis co-advisor, Assistant Professor Rujipat Samransamruajkit, for his valuable suggestion, support and encouragement for the completeness this thesis. I am very grateful to my supervisory committee, Associate Professor Dr. Kanisak Oraveerakul, Associate Professor Dr. Chantapong Wasi, Associate Professor Dr. Parvapan Bhattarakosol for their valuable suggestion and criticisms.

My sincere appreciation is also expresses to Professor Dr. Peter Simmonds for his valuable advice, excellent ideas and kindness support during one year of my research study at the Center for Infectious Diseases, University of Edinburgh, UK. Special thank must also be extended to Dr. Jill Douglas and Dr. Carol Leitch for their advices and suggestion.

I am indebted to Ms. Apiradee Theamboonlers, Ms. Chitima Thongmee, Ms. Kesmanee Praianantthavovn, Ms.Soraya Panklom for their technical instructions and sincere helpful during my study. I would like to thank Dr.Sunchai Payunporn, Mr. Kamol Suwanakarn, Ms. Woradee Lurchachaiwong, Ms Pattaratida Sa-nguanmoo, Ms Srunthron Akkarathamrongsin for their assistances of some materials and logistic works.

I am particular indebted to the Royal Golden Jubilee (RGJ) Ph.D. Program, Thailand Research Fund (TRF) and the Commission on Higher Education, Ministry of Education, Thailand. Special appreciation must be also be extended to the Center of Excellent in Clinical Virology, Department of Paediatics, Faculty of Medicine, Chulalongkorn University and Center for Infectious Diseases, University of Edinburgh, UK. Finally, I would like to express the profound gratitude to my beloved parents, family and friends for their love, encouragements and moral supports throughout this study.

CONTENTS

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF ABBREVIATIONS

μg	=	Microgram
μl	=	Microlitre
μM	=	Micromolar
Adeno	=	Adenovirus
AFP	=	Acute flaccid paralysis
BLAST	=	Basic local alignment search tool
BPV	=	Bovine parvovirus
BSA	=	Bovine serum albumin
CMV	=	Canine minute virus
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylene diamine tetraacetic acid
EM	=	Electron microscope
FluA	=	Influenza A virus
FluB	=	Influenza B virus
GAPDH	=	Glyceraldehyde-3-phophate dehydrogenase
GI	=	Gastrointestinal
HBoV	=	Human bocavirus
HBoV2	=	Human bocavirus 2
HCoV	=	Human coronavirus
hMPV	=	Human metapneumovirus
hr	=	Hour
Min	=	Minute
ML	=	Maximum likelihood
NCBI	=	National Center for Biotechnology Information
NP	=	Nasopharyngeal
NP1	=	Nonstructural protein with unknown function
NS1	=	Nonstructural protein 1
nt	=	Nucleotide
ORFs	=	Open reading frames

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ParaFlu	=	Parainfluenza virus
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
rpm	=	Round per minute
RSV	=	Respiratory syncytial virus
RT	=	Reverse transcription
RT-PCR	=	Reverse transcription-Polymerase chain reaction
sec	=	Second
UK	=	United Kingdom
VP1	=	Viral capsid protein 1
VP1u	=	Viral capsid protein 1 unique region
VP2	=	Viral capsid protein 2

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Acute respiratory tract infection is a major cause of pediatric morbidity and mortality worldwide. The clinical feature of respiratory infection is obviously presented but the etiological agent is more difficult to identify. In most cases, respiratory viruses including influenza A and B viruses, parainfluenza viruses, adenoviruses, and respiratory syncytial viruses (RSV) are causative agents. By using the molecular based method, many viruses had been detected from the respiratory tracts including human metapneumovirus (hMPV), human coronavirus (HCoV)-229E, HCoV-OE43, HCoV-HKU1, and also human bocavirus (HBoV), all of which association to the upper and lower respiratory tract infections.

Recently, a new respiratory tract virus of the *Parvoviridae* family, Human Bocavirus (HBoV), has been discovered by applying molecular analysis on pooled respiratory tract aspirations taken from children in Sweden by Allander T *et.al.* and colleague in 2005 [1]. This virus is closely related to the bovine parvovirus (BPV) and canine minute virus (CMV), which has been classified as members of the genus *Bocavirus.* The virus composes of two major open reading frames (ORFs) encoding a nonstructural protein (NS1) and at least two capsid proteins (VP1 and VP2). Moreover, the HBoV genome also contains a third middle ORF encoding a nonstructural protein (NP1) of unknown function [1]. The most conserved region of this virus is the NS1 and NP1 gene whereas the VP1/VP2 gene constitutes the variable region [2]. Due to lack of propagation techniques in cell culture or animal models, the pathogenesis of respiratory illness by HBoV is not clearly defined. However, many studies have reported that HBoV was associated with acute respiratory illness [1-3] Upon discovery of HBoV in respiratory pools, its global prevalence has been reported to range from 0.8% to 19% and co-infection with other viruses was commonly found [1, 3-24]. Moreover, few studies

have shown negative results for HBoV infection in nasopharyngeal (NP) swabs from healthy subjects [20, 25-26]. Not only HBoV can be detected in the respiratory tract, but also in the gastrointestinal (GI) tract had been reported in Spain, Brazil, Korea and Hong Kong [27-30]. All data presented thus far had exclusively originated from children with acute diarrhea. The additional epidemiological and clinical investigation are essential in order to elucidate what exactly HBoV related illness. Therefore, in the present study the polymerase chain reaction was applied to detect HBoV from NP aspirations collected from infants and children who had been admitted with respiratory tract illness and stool collected from children hospitalized with acute gastroenteritis in comparison with healthy children serving as controls. Moreover, the newly discovered species of *Bocaviruses* also had been reported in this study.

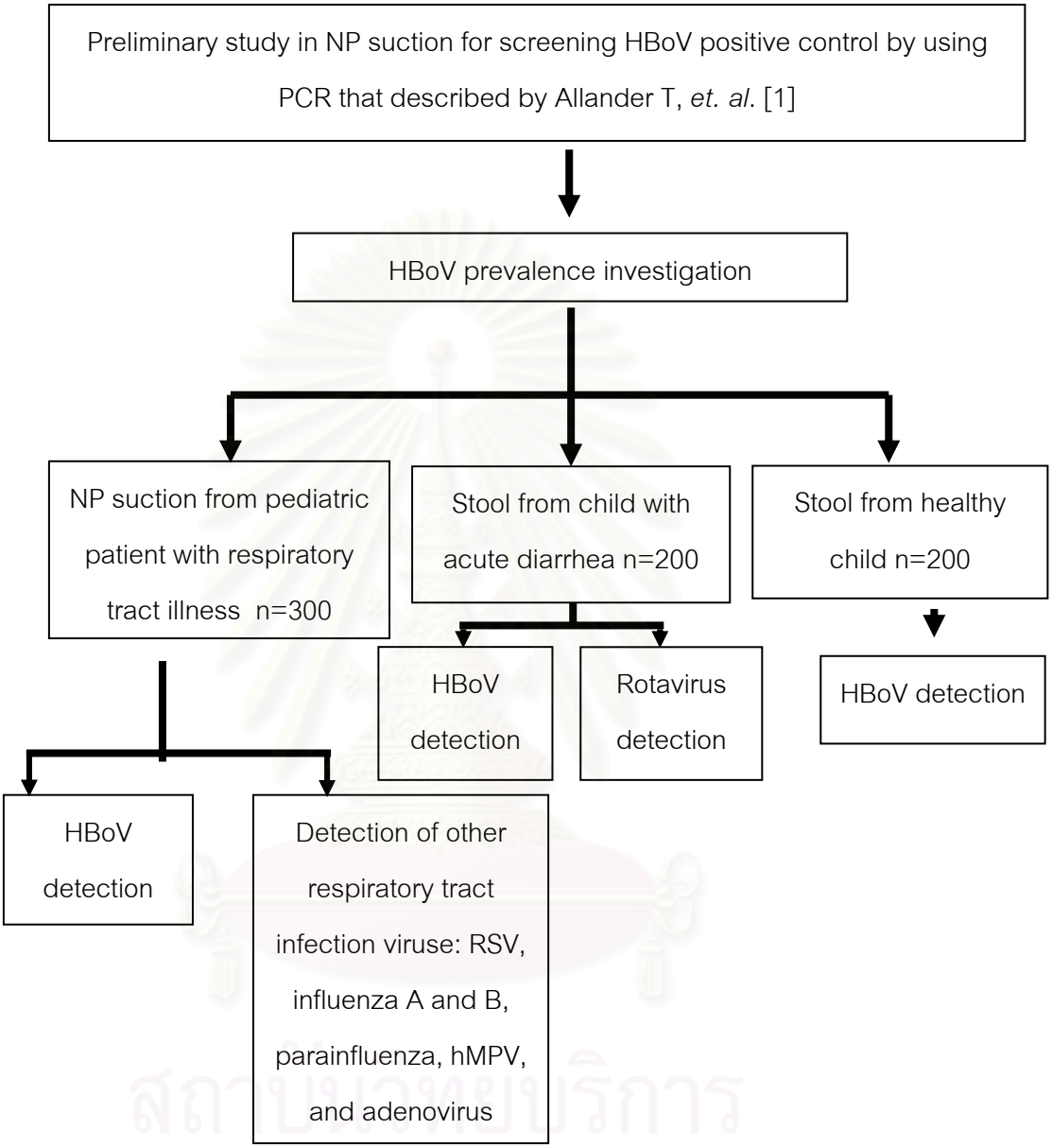
Objective

1. To comparison the sequences of HBoV in Thai children and globalize by complete coding sequences analysis.
2. To investigate the prevalence of HBoV in NP suction of Thai pediatric patients who have respiratory tract infection and stool from children with acute diarrhea.
3. To investigate the association between HBoV infection and healthy children stool samples.
4. To identify new HBoV2 in Thai and UK samples

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Experimental Design

HBoV study



สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

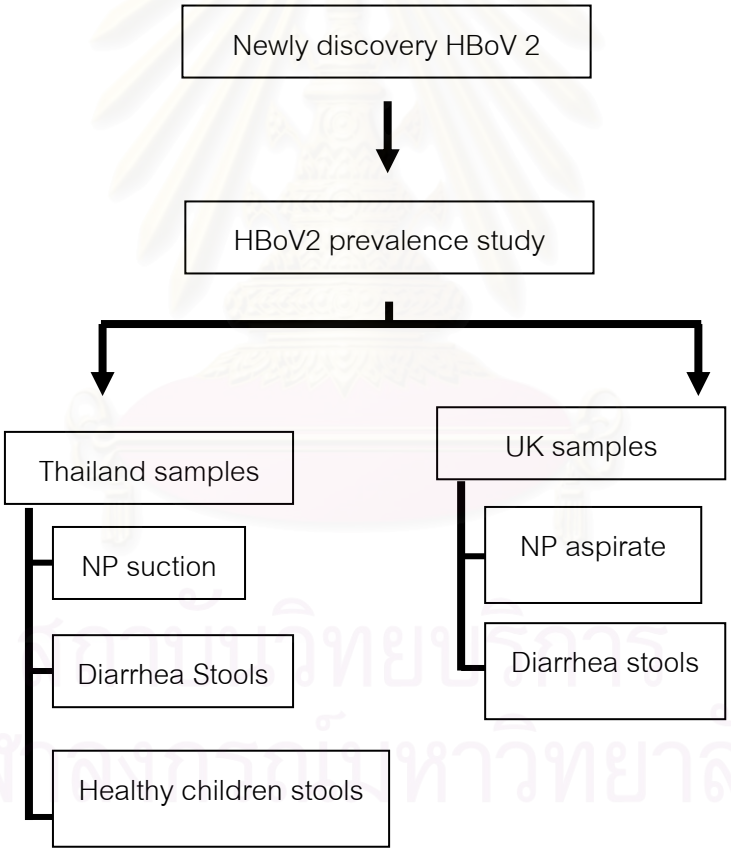
HBoV study (continue)

Preliminary study in NP suction for screening HBoV positive control by using PCR that described by Allander T, *et. al*. [1]

↓

Three HBoV selected from NP aspirate and two HBoV from diarrhea stools for complete coding sequences analysis.

HBoV2 study



ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Expected Benefits

- The study will provide novel knowledge of HBoV and HBoV2 in respiratory and gastrointestinal tracts infections, from which it will be applied for diagnosis, prevention, clinical outcome and propose management in HBoV and HBoV2 infected patients.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

REVIEW AND RELATED LITERATURES

Respiratory Virus Infection

Respiratory virus infection is the major public health problem because of the worldwide spreading and high mobility and mortality in children. Respiratory illnesses occur in all age, however, children are infected 2-3 times more than adults. Approximately 5 million children less than 5 years did died by respiratory infection in developing countries [31]. Respiratory virus infections are also associated with considerable costs in terms of decreased productivity and time loss from work or school, visit to health care providers, and the amount of drug prescribed. Viruses that cause acute respiratory illness mostly transmitted via direct contract or droplets although some are transmitted by aerosol. Viruses that commonly found in patients include influenza A virus, influenza B virus, RSV, parainfluenza viruses and adenovirus.

The etiological diagnosis of viral respiratory tract illness requires the laboratory confirmation and the recognition of symptoms and signs. Traditionally diagnoses of these viruses are isolation and identification techniques in cell cultures and detection of viral antigen by direct immonufluorencent assay. Recently, the polymerase chain reaction (PCR) base assay has been introduced to use as the viral respiratory pathogens detection method. Because of the sensitivity of this method is high, therefore, many viruses that shown the association of respiratory illness had been identified such as hMPV [32], HCoV-NL63 [33], HCoV-HKU1 [34], and also HBoV [1].

HBoV Discovery and Classification

HBoV has been firstly described in 2005 by using the molecular virus screening by Allander T *et al.* [1]. The pools of respiratory aspirates had been making up from the samples that randomly collect in Stockholm, Sweden. The nucleic acid had been extracted after processing and treats with DNase and RNase. After the random

amplification, cloning and following by large scale sequencing and bioinformatic analysis had been used, Parvovirus-like sequences were found. These nucleotide sequences showed no significant similarity to NCBI database in the BLAST search. However, the amino acid sequences significantly matched to those of bovine parvovirus (BPV) and canine minute virus (CMV) two related members of *Parvoviridea* family, subfamily *Parvovirinea*, genus *Bocavirus*. After performing the complete genome analysis, the new virus shown the clearly separating from BPV and CMV in which having only 42%-43% amino acid identity to the nearly neighbor CMV from phylogenetic analysis (Fig 1). Therefore, this study group concludes that this virus is the new uncharacterized species of genus *Bocavirus* and the name was proposed as human bocavirus or HBoV [1].

The genome organization of HBoV closely related to the other member of *Bocavirus* genus BPV and CMV (Fig 2). The viral genomes comprised of 5,217 nucleotide (Stockholm 1; ST1 isolate, DQ000495) and there are two major open reading frames (ORFs) encoding nonstructural protein (NS1) and at least 2 capsid proteins (VP1 and VP2), respectively. Parvoviruses normally encode two capsid proteins VP1 and VP2 that are identical in sequences but only differ in the amino acid-terminal extension of VP1 at N-terminal named as VP1 unique region (VP1u). In HBoV and other parvoviruses such as pavovirus-B19 and CMV, the VP1u has the phospholipase A2-like activity (PLA2). This viral PLA2 played the role for viral entry step that mediated the transfer of viral genome from endocytotic component to the host cell nucleus [35]. Moreover, HBoV also has a third ORF between NS1 and VP1 which encoding nonstructural protein with unknown function (NP1) that sharing 47% amino acid identity to NP1 of CMV and BPV.

Since this virus has been identified, it could not be propagated in any cell culture or animal model. Therefore, the Koch's postulate still could be not fulfilled in this virus. However, recently, the HBoV had been detected by electron microscopy (EM) in nasopharyngeal (NP) aspirates from patients that positive HBoV DNA. The EM results shown the hexagonally shaped defied with approximately 25 nm in diameter in which are the typical structural characteristics of parvoviruses (Fig 3) [36].

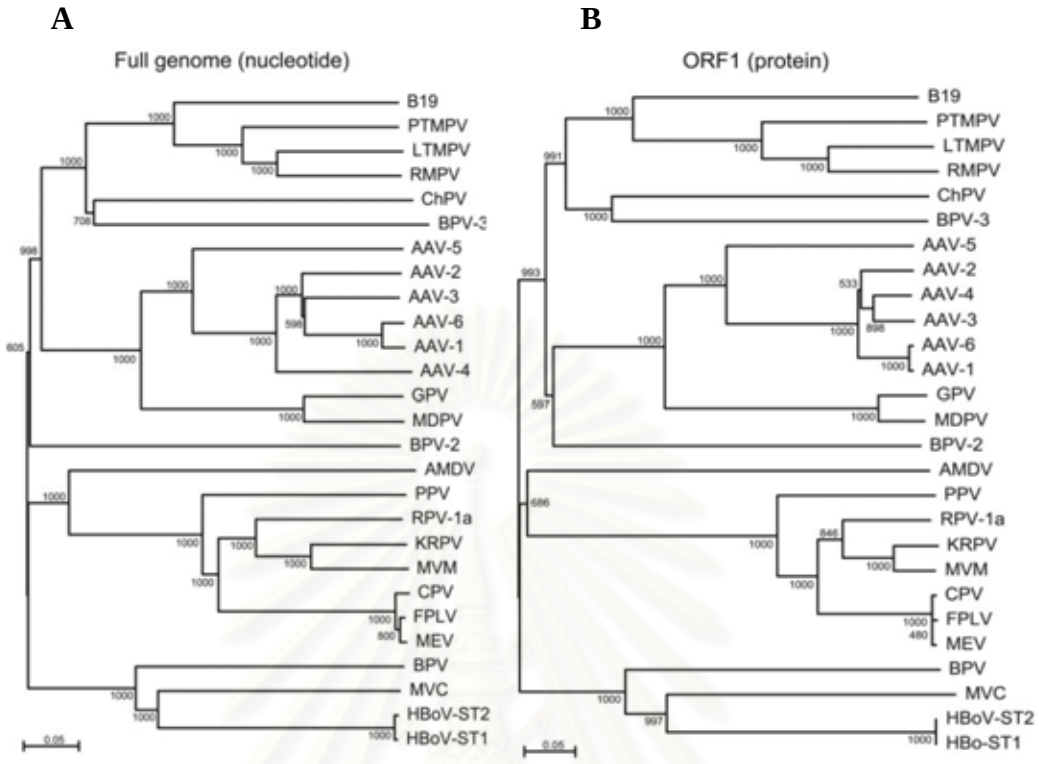


Figure 1. Phylogenetic analysis of HBoV compared with the other members of *Parvoviridea* family viruses: bootstrapped neighbor-joining tree based on full-length nucleotide sequences (A) and ORF1 amino acid sequences (B) of HBoV and the *Parvovirinae*. Bootstrap values are indicated at each branching point. Analysis of capsid gene nucleotide and amino acid sequences yielded highly similar results (data not shown). B19, erythrovirus B19; PTMPV, pig-tailed macaque parvovirus; LTMPV, long-tailed macaque parvovirus; RMPV, rhesus macaque parvovirus; ChPV, chipmunk parvovirus; AAV, adeno-associated virus; GPV, goose parvovirus; MDPV, Muscovy duck parvovirus; AMDV, Aleutian mink disease virus; PPV, porcine parvovirus; RPV-1a, rat parvovirus-1a; KRPV, Kilham rat parvovirus; MVM, minute virus of mice; CPV, canine parvovirus; FPLV, feline panleukopenia virus; MEV, mink enteritis virus. [1]

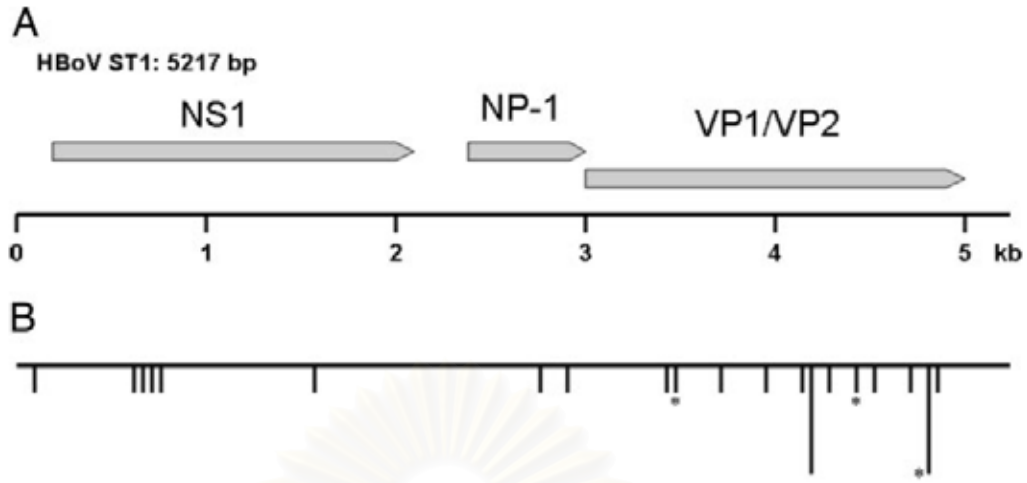


Figure 2. HBoV genome organization. The schematic maps showed the genome organization of HBoV (A) and the location of the difference nucleotides of two isolates of HBoV: ST1 and ST2 (B). The horizontal line represents the sequence of ST1, and each vertical line represents a nucleotide difference to ST2. In two cases where several differences were located close together, a longer vertical line representing four differences. The asterisks mark the three differences that resulted in a predicted amino acid change. [1]

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

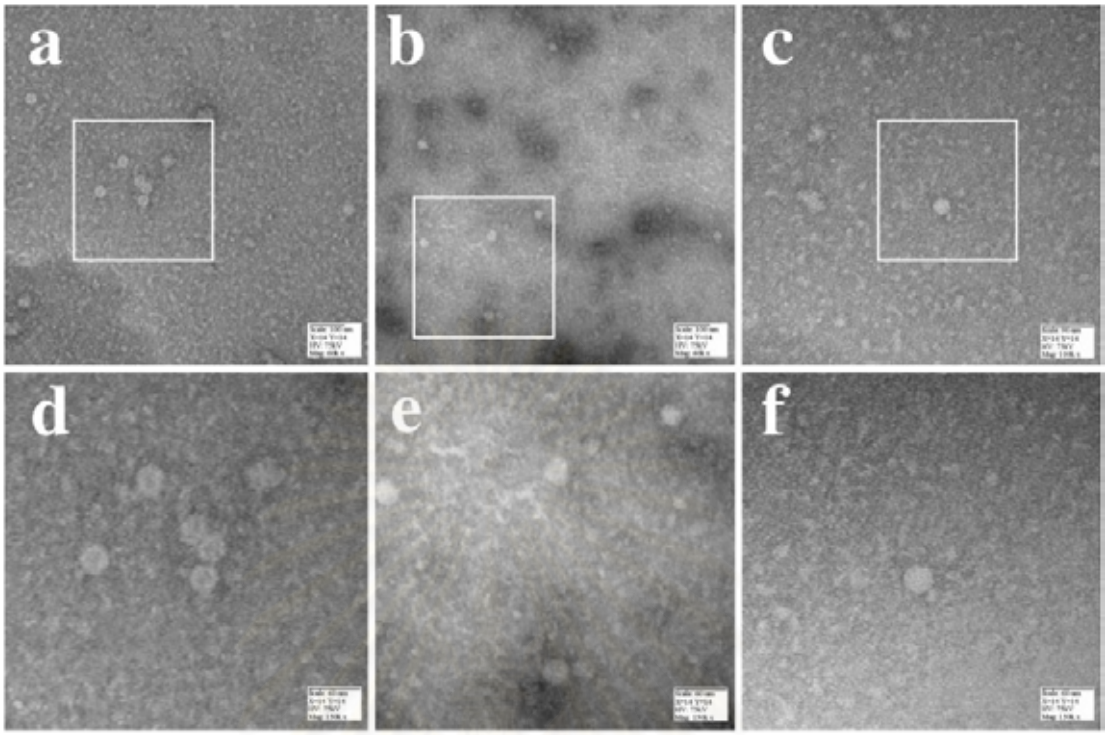


Figure 3. The EM of HBoV that published by Brieu N *et al.* [36]. a and d, b and e, and c and f are the EM result of HBoV in three different samples after negative staining with 4% uranyl acetate, observed at x60,000 (a and b), x100,000 (c), and x150,000 magnification (d, e, and f).

The Epidemiology Study of HBoV

Since it the first report in 2005, HBoV has been frequently detected worldwide (Fig 4). Most study was investigated in respiratory secretion in pediatrics patients with respiratory tract diseases and the prevalence of HBoV infection has been reported. It varied from 1.5-19% depend on type of samples and detection methods that shown in Table 1. Moreover, in 2007, the detection of HBoV DNA in enteric samples had been reported and the following studies showed that the prevalent rate was between 0.8-9.1% (Table 1). HBoV detection mostly found in winter season in many study, however, few studies also reported the increasing numbers of HBoV infection in summer or spring.

Therefore, these result indicated that there was no regular seasonal occurrence of this virus infection.

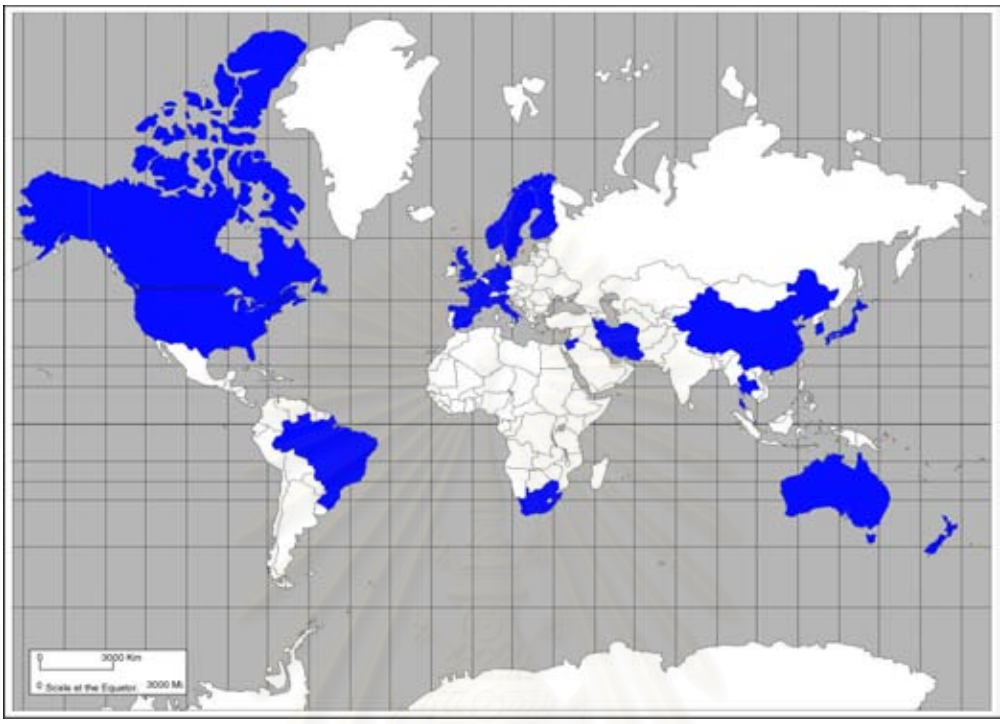


Figure 4. The global HBoV epidemiologic study. The HBoV detection had been reported in countries that marked in blue

HBoV infection usually detected in children less than 3 years old but low prevalence among youngest children below 6 month and adult. Because of the lack of animal model and still can not propagated in any cell culture, the detail of seroprevalence has been limited. However, the virus like particle that expressed in vitro had been used as the antigen to detect the antibody of HBoV in term of to fulfill the detail of seroepidemiolgy. The VP1 and VP2 have been selected for virus like particle production in American [37] and Japanese [38] groups, respectively. In both studies shown the same result, about 90% of infant younger than 2 years old had highly seropositive for HBoV from which may represent the acquired maternally antibody and were drop in lowest seropositive in age range between 6 and 8 month. After that the seroprevalance rapidly increasing and completely immunize (100%) in about over 6

year-age group. These results are supported that HBoV DNA usually detected in samples from patient between 6 month and 3 years old.

The genetic variation of HBoV is very low. Many studies report the high level of genomic conservation of this virus in global detection [30, 39]. Phylogentic analysis indicated that two slightly different genetic lineage circulate around the world in all type of samples [25].

Table 1 Summary of HBoV epidemiology study

Country	Type of samples	% positive	Reference
South Korea	NP suction	8.0	[8]
Hong Kong	NPAs	5.0	[30]
	Fecal	2.1	
Sweden	NPAs	3.1	[1]
Spain	NPAs	7.1	[40]
Canada	NP swabs	5.1	[4]
Japan	NP swabs	5.7	[11]
South Korea	NPAs	11.3	[41]
UK	NPAs	8.2	[12]
USA (Ohio)	NPAs	2.3	[42]
Australia	NPAs	5.6	[14]
German	NPAs	10.3	[16]
France	NPAs with KD	5.5	[43]
Australia	NPAs	4.8	[5]
Spain	NPAs	13.4	[44]
Israel	NPAs	11.3	[45]

NPAs: Nasopharyngeal aspirates; NP: Nasopharyngeal; KD: Kawazaki Disease

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 1 (Continue) Summary of HBoV epidemiology study

Country	Type of samples	% positive	Reference
Thailand	Swab from out patient	1.0	[18]
	Swab from patient with fever	4.5	
	Swab from URTI case	3.9	
Finland	NPAs with wheezing	19	[17]
	Swab from Asymptomatic	0	
China	NPAs	4.8	[46]
France	NPAs	3.4	[47]
Netherlands	NP swab	1.6	[21]
France	NPAs	4.4	[9]
South Africa	NPAs	11	[15]
New Zealand	Thawed, NPAs	3.5	[48]
Iran	NPAs or swab	8.0	[22]
Italy	swab from patient	4.5	[20]
	swab from healthy person	0	
Jordan	NPAs	18.3	[10]
USA	NPAs from patient	5.2	[25]
	Swab from asymptomatic	0	
China	NPAs	8.3	[23]
Canada	Swab, NPAs	1.5	[6]
Canada	NPAs from adult patients	0.8	[49]
	NPAs from children patients	13.8	
France	NPAs	12.5	[50]
German	NPAs	2.8	[51]
USA	NPAs	5.6	[3]
South Africa	NP Swab with acute wheezing	7.4	[52]
South Korea	NPAs	11.3	[7]
Singapore	NP Swab	8.0	[53]

NPAs: Nasopharyngeal aspirates; NP: Nasopharyngeal; KD: Kawazaki Disease

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 1 (Continue) Summary of HBoV epidemiology study

Country	Type of samples	% positive	Reference
South Korea	Stool	0.8	[28]
Spain	Stool	9.1	[27]
	NPAs	7.7	
China	Stool from diarrhea cases	3.5	[54]
	Stool from asymptomatic cases	3.5	
China	Stool	5.5	[55]
Brazil	Stool	2.0	[29]
German	Stool	4.6	[56]

NPAs: Nasopharyngeal aspirates; NP: Nasopharyngeal; KD: Kawazaki Disease

The Association of Clinical Manifestation and HBoV Infection

Many world-wide epidemiology study had been reported the symptom that usually appear with HBoV infection, however, the highly co-infection rate with other respiratory viruses also had been reported [1, 3, 4, 6-34, 39-42]. Therefore, it is difficultly to conclude the pathogenicity of this virus infection. Moreover, because of the Koch's postulates still can not be fulfilled, the disease causality of this virus infection also have not been clarified. However, in 1960, Fredricks and Relman [57] had been proposed the alternative guidelines for establishing microbial disease causation base on molecular diagnosis in the absence of a cultivated or purify microorganism. They proposed the following guidelines:

(i). A nucleic acid sequence belonging to a putative pathogen should be present in most case of an infectious disease. The microbial nucleic acid should be found at the sites that has pathogenicity and not in the sites that lack of pathogenicity.

(ii). Fewer, or not, copy numbers of pathogen-associated nucleic acid sequences should be not found in tissue or host without disease.

(iii). The copy number of nucleic acid should be correlated with the clinical outcome for example, the copy number of nucleic acid should be decreased or undetectable when has the clinically effective treatment and with clinical relapse, the opposite should occur.

(iv). When sequence detection in predate disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be causal relationship.

(v). The nature of the microorganisam inferred from the available sequence should be consistent with the known biological characteristics of that group of organism.

(vi). Tissue-sequence correlates should be sought at the cellular level in which the specific in situ-hybidization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganism are presumed to be located.

(vii). These evidences for microbial causation should be reproducible.

Therefore, this guideline seems to be the best model for conclude the clinical association of HBoV infection.

The disease induction of HBoV infection still can not be concluded, although, the association between HBoV DNA detection and upper respiratory tract infection (URTI) and also lower respiratory tract infection (LRTI) had been reported [1, 12, 15, 21, 40]. The symptom including fever, cough, wheezing, rhinorhea and diarrhea are commonly found in children that positive HBoV DNA. Many recently studies reporting the association of HBoV and disease appearance also including the asymptomatic control population group [17, 18, 20, 25]. The result show that HBoV highly detected in patient group, whereas the virus either not found or was found in small number in control groups. However, the difference of samples types, age group, and period samples collection time should be underlined for conclude the result from asymptomatic group.

Human Bocavirus2 Discovery

The newly species of human bocavirus species had been identified from the stool samples of Pakistani child with acute flaccid paralysis (AFP). After using non specific viral-particle purification, random nucleic acid amplification and minimal sequencing, the BLAST program was used and the results show that the sequences highly significant with the HBoV reference genome (NC_007455). The total 5,196 bases of newly identified HBoV genome were assembly by the PCR, and 5'RACE and 3'RACE as the method that previously described [58]. Because of the genome closest related of this newly identified virus to HBoV, therefore, it had been named as "Human bocavirus 2 (HBoV2)".

The genome organization of HBoV2 is similar to HBoV in which containing with 3 large ORF (Fig 5). The first 5'ORF is NS1, is important for viral DNA replication and gene expression, its show 78% of the amino acid identity with HBoV. The second ORF is NP1, it was 4 amino acids shorter and show 67% identity to HBoV. The third ORF is VP1/VP2 that enclosed a protein with 80% identity to the HBoV. The VP1 of HBoV2 was preceded by 25 amino acid methionine-initiated ORF stretch and 4 amino acid deleted downstream, therefore it slightly larger than VP1 in HBoV. By comparing with VP2 of HBoV, VP2 protein of HBoV2 should be started at the 154 amino acid of the third large ORF.

The phylogenetic analysis was used to determine the relationship between HBoV2 and other member of *Bocavirus* genus. All three ORF genome sequences and deduced amino acid sequences of HBoV2 prototype (PK5510) are closely related to HBoV than CMV and BVP. The pairwise nucleotide distances between HBoV2 and HBoV, which shown in 22%-26%, were substantially greater than those within the HBoV clade (0.4%-0.9%) but were less than those between either the HBoV or the HBoV2 sequence and animal bocaviruses (46%-56%). At this first investigation, HBoV2 variants were distributed among 3 groups or genotype from phylogenetic tree in which the genome show 4.5% sequences divergent in each other: genotype 1, HBoV2 prototype PK5510; genotype 2, PK2255; and genotype 3, UK-648. The diversity of three genotype

investigation result was shown in phylogenetic tree (Fig 6) and sliding window analysis (Fig 7). The VP1 of genotype 1-PK5510 and genotype 2-PK2255 were closely clustered in which genotype 3-UK648 were outlier. And the sliding window also shows the identical between genotype 1 and genotype 2 of HBoV2 in this VP1 region which is the green line in Fig 7. The NP1 and NS1, whereas, were closely clustered in genotype 2-PK2255 and genotype 3-UK648 and the genotype 1-PK5510 was the outlier.

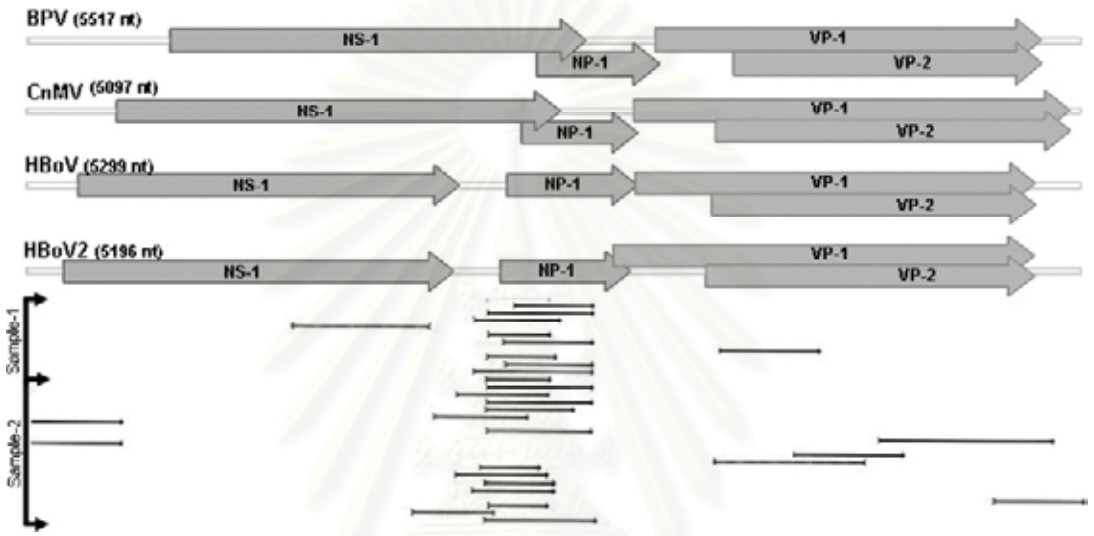


Figure 5. Schematic representation of genomic organization of HBoV2 and other species of bocaviruses. The location of viral sequences obtained by shotgun sequencing in the first and second stool samples are indicated by the horizontal lines. BPV, bovine parvovirus; CnMV, canine minute virus; HBoV, human bocavirus; HBoV2, newly identified parvovirus species.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

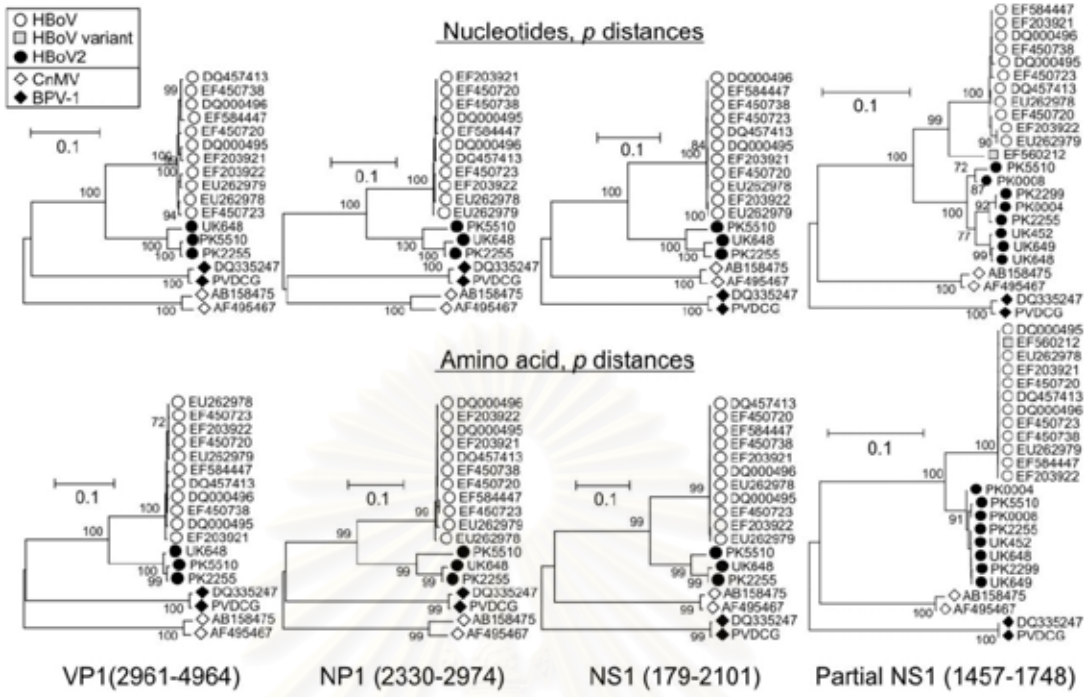


Figure 6. Phylogenetic analysis of HBoV and HBoV2. The 3 major open reading frames were analyzed on the basis of both nucleotide and protein sequences of representative variants of human bocavirus (HBoV), HBoV2, CnMV (canine minute virus), and bovine parvovirus 1 (BPV1). Analysis of the partial NS1 sequence was used to show phylogenetic relationships between a larger number of samples amplified by polymerase chain reaction; this sequence also corresponded to the region of the partially sequenced Brazilian HBoV variant (EF560212). HBoV2, newly identified parvovirus species.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

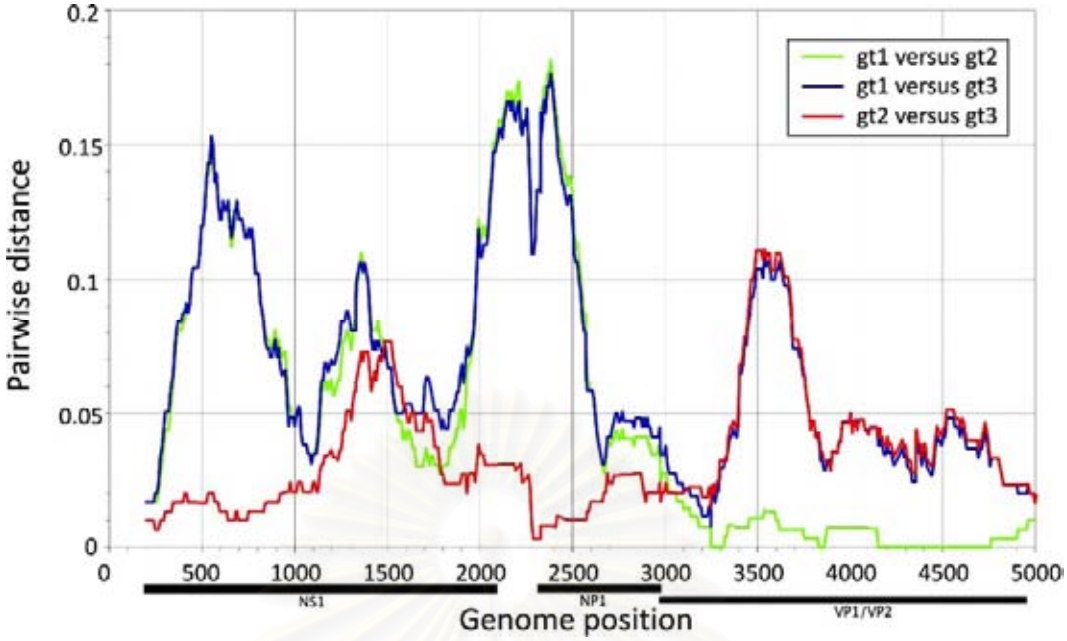


Figure 7. The sliding window analysis of HBoV2. Divergence between pairs of nearly complete nucleotide genome sequences of HBoV2 genotypes. Sequences in the partial NS1 regions of 3 different genotypes; namely, genotype 1 (gt1), PK5510; genotype 2 (gt2), PK2255; and genotype 3 (gt3), UK648; were aligned to determine sequence divergence (uncorrected p distances), by use of a window size of 300 and 9 base increments. Sharp transitions in sequence divergence between gt1 and gt2 and between gt2 and gt3 indicate possible breakpoints associated with recombination events.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III

MATERAILS AND METHODS

Clinical Samples

Thailand samples

- Nasopharyngeal (NP) Suction

The specimens were collected from 302 infants or children (age: 5 days to 14 years) who admitted and diagnosed as the acute lower respiratory illness during February 14, 2006 to February 28, 2007. All of clinical samples were provided by the Department of Pediatrics, King Chulalongkorn Memorial Hospital, Thailand. NP suction samples were collected in transport medium with antibiotics (0.5% BSA, Penicillin G ($2x10^6$ U/L), Streptomycin 200 mg/liter) and stored at -70^{o}C until tested.

- Stool Samples

The samples were collected from two population groups. The first one comprised of 225 children from 4 months to 4 years of age who had been admitted to the King Chulalongkorn Memorial Hospital, Bangkok and Buriram Provincial Hospital, and diagnosed with acute diarrhea between November 2005 and September, 2006. Another is the control group consisted of 202 healthy children aged between 2 months and 5 years from the well baby clinic, King Chulalongkorn Memorial Hospital and Kindergarten school in Bangkok whose parents had collected their stool samples from May to September, 2007. All stool samples were diluted 1:10 in PBS, thoroughly mixed on a vortex and centrifuged at 8,000 rpm for 10 minutes. Supernatants were collected and stored at -70^{o}C until tested.

Edinburgh, UK samples

- NP aspirate samples

The study was based on a total of 6,138 respiratory samples in which collected from January 1, 2007 to June 30, 2008, referred to the Specialist Virology Centre (SVC), Royal Infirmary of Edinburgh.

- Stool Samples

2,500 samples were collected from Enteric Lab, Western General Hospital, Edinburgh, UK in March, June and September 2008. All stool samples were diluted 1:10 in PBS, thoroughly mixed on a vortex and centrifuged at 8,000 rpm for 10 minutes. Supernatants were collected and made up the pool of ten samples then stored at -20°C until tested.

DNA and RNA Extraction

-TRI REAGENT®LS method

In Thailand samples, DNA and RNA were extracted from 150 µl of NP suction or stool suspension using TRI REAGENT® LS (Molecular Research Center, Inc., Cincinnati, OH) and the pellet at the final step had been solubilized in 20 µl of 8 mM NaOH or 12 µl of DepC treated water for DNA or RNA, respectively.

- MinElute virus spin kit method

In UK samples, DNA and RNA were extracted from 140 µl of clinical specimens by using a MinElute virus spin kit and eluted into 40 µl Tris-EDTA buffer according to the manufacturer's instructions (Qiagen,Hemel Hempstead, UK). Extracted DNA and RNA were stored at -20°C. Pools of 10 samples were made up for subjected to extraction.

Reverse Transcription

Reverse transcription (RT) was performed at 37°C for 2 hr using 200 units of M-MLV reverse-trancriptase (Promega, Madison, WI), 5 µl of 5X M-MLV reaction buffer (Promega, Madison, WI), 5µl of 10mM dNTP (Promega, Madison, WI), 25 units of rRnasin® Ribonuclease Inhibitor (Promega, Madison, WI) and 0.5µg/µl of Random Primer (Promega, Madison, WI), 12 µl of RNA heating to 70°C for 5 min then cooling on ice, and adding nuclease-free water to a final volume of 25 µl. The cDNA was served as the template for RNA virus detection.

HBoV Detection

The NP1 gene was amplified by conventional PCR modified from a previous study [1]. The reaction mixture contained 2 µl DNA, 0.5 µM 188F primers: 5'-GAGCTCTGTAAGTACTATTAC-3'(nt 2351-2371)and 0.5 µM 542R primer: 5'-CTCTGTGTTGACTGAATACAG-3' (nt 2704-2684), 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: Initial denaturation at 94ºC for 3 min, followed by 35 amplification cycles consisting of 94°C for 30 sec (denaturation), 55°C for 30 sec (primer annealing), and 72°C for 1 min (extension), and concluded by a final extension step at 72°C for 7 min. Another set of primers specific for the VP1 gene, VPF2 forward primer: 5'-TTCAGAATGGTCACCTCTACA-3' (nt. 3,639-3,659) and VPR2 reverse primer: 5'-CTGTGCTTCCGTTTTGTCTTA-3' (nt. 4,286-4,266), were used in a separate PCR reaction to exclude false positives. After 2% agarose gel electrophoresis stained with ethidium bromide, the expected products of 354 and 648 bp representing the NP1 and VP1 gene, respectively, were visualized on a UV transilluminator.

HBoV2 Detection

Nested PCR was used as the detection method. The first round PCR, raction mixture containing of 2 µl DNA, 0.5 µM Boca2-sf1 primer 5'-AACAGATGGGCAAGCAGAAC-3' (nt 1,484 – 1,504) and 0.5 µM Boca2-sr1 5'-AGGACAAAGGTCTCCAAGAGG-3' (nt 2,031-2,052) 0.03 mM dNTP (Promega, Madison,

WI), 0.02 U of GoTaq™ (Promega, Madison, WI), 4 µl of 10X GoTaq™ reagent buffer (Promega, Madison, WI), and nuclease-free water to a final volume of 20 µl. The amplification was performed by following condition: 95°C for 3 min, 5 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min, and following by 35 cycles of 95°C for 30 sec, 51°C for 30 sec, and 72°C for 45 sec, then final conclude by 72°C for 10 min. After that 1 µl of first round PCR products will be subjected to second round PCR. The reaction mixtures are containing with 0.5 µM HBoV2_1618S primer 5'-AACGATTGCAGACAACGCCTTATA-3' (nt 1,618 – 1,642) and 0.5 µM Boca2-sr2 5'-TCCAAGAGGAAATGAGTTTGG-3' (nt 2,019 – 2,030), 0.03 mM dNTP (Promega, Madison, WI),0.02 U of GoTaq™ (Promega, Madison, WI), 4 µl of 10X GoTaq™ reagent buffer (Promega, Madison, WI),and nuclease-free water to a final volume of 20 µl. The amplification was performed by following condition: 95°C for 2 min, 5 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and following by 35 cycles of 95°C for 30 sec, 54°C for 30 sec, and 72°C for 45 sec, then final conclude by 72°C for 10 min. The expected band should be shown in 413 bp when vitalizing by UV transilluminator after running though the 2% agarose gel electrophoresis and staining with ethidium bromide.

Adenovirus Detection

The primers that designed for subgroup B and E amplification were used by following previously published method [59]. The real-time PCR by using SYBR Green I had been used as the detection method. The reaction mixture contained 1 µl of DNA sample, 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), 0.5 µM AdenoF: 5'-GATGGCCACCCCATCGATGMTG-3' (nt.1-22), 0.5 µM AdenoR: 5'-GCGAACTGCACCAGACCCGGAC-3' (nt. 95-74) , 2 µl 1X SYBR Green and nuclease-free water to a final volume of 20µl. The reaction mixtures were subjected into Rotor-Gene 3000 real-time PCR machine (Corbett Research, New South Wales, Australia). The amplification reaction consisted of a pre-incubation step at 95°C for 3 min followed by 35 cycles of amplification including 95°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec. The fluorescent signal was detected once per cycle upon completion of the extension step. After amplification, melting curve analysis was performed by heating to

95°C then cooling to 60°C for 15 sec, followed by a temperature increase to 95°C, while continuously collecting the fluorescent signal data.

Influenza A Virus Detection

The detection was performed by conventional PCR using 1 µl of cDNA, 0.5 µM of FluA_M_F: 5'-RGGCCCCCTCAAAGCCGA-3' (nt 76-93), 0.5 µM of FluA_M_R: 5'-ACTGGGCACGGTGAGYGT-3' (nt 235-218), 10 µl of 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: Initial denaturation at 94ºC for 3 min, followed by 40 amplification cycles consisting of 94°C for 30 sec (denaturation), 55°C for 30 sec (primer annealing), and 72°C for 1 min (extension), and concluded by a final extension step at 72°C for 7 min. After 2% agarose gel electrophoresis, the expected PCR product was shown in 160 bp.

Influenza B Virus Detection

The matrix gene of influenza B virus was amplified by using the conventional PCR method. The reaction mixture contained 2 µl cDNA, 0.5 µM of the forward and reverse primer were used by following the previous published method [60] :FluBMF: 5'-ATGTCGCTGTTTGGAGACACAAT-3' (nt 25-47), and FluBMR: 5'-TCAGCTAGAATCAGRCCYTTCTT-3' (nt 320-301), 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 94ºC for 3 min, followed by 35 amplification cycles consisting of 94°C for 30 sec (denaturation), 55°C for 30 sec (primer annealing), and 72°C for 1 min (extension), and concluded by a final extension step at 72°C for 7 min. After 2% agarose gel electrophoresis, the expected PCR product was shown in 295 bp.

Parainfluenza Virus Detection

Real-time PCR using SYBR green I carried out in a Rotor-Gene 3000 (Corbett Research, New South Wales, Australia) was used for subtype 1-3 of this virus detection. The reaction mixture contained 1 µl of cDNA sample, 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), 0.5 µM ParaF: 5'-GCTAAATACTGTCTTMAHTGGAGAT-3' (nt. 11,254-11,278), 0.5 µM ParaR: 5'-GTAAGGATCACCWACATADAWTGTA-3' (nt. 11,392-11,370) , 2 µl 1X SYBR Green and nuclease-free water to a final volume of 20µl. The amplification reaction consisted of a pre-incubation step at 95°C for 3 min followed by 35 cycles of amplification including 95°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec. The fluorescent signal was detected once per cycle upon completion of the extension step. After amplification, melting curve analysis was performed by heating to 95°C then cooling to 60°C for 15 sec, followed by a temperature increase to 95°C, while continuously collecting the fluorescent signal data.

Human Metapneumovirus (hMPV) Detection

The nested PCR detection method described by Thanasugarn *et al*.[61] was used. Briefly, the first PCR amplification performed in a total volume of 25 µl containing 2 µl of cDNA, 0.5 µM of MPVPF: 5'-ACGGGGTAGAGAAGAGCTGG-3' (nt 389–408) and MPVPR: 5'-GCAAAGTTGGGACAGTTGGC-3' (nt 1004–985), 10 µl of 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The PCR conditions comprised one initial denaturation cycle at 94 °C for 1 min, followed by 35 cycles at 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 7 min. The first PCR product had been further amplified using the inner forward MPVNF: 5'-GCATCAACCATAGAAGTGGGAC-3' (nt 556–577) and reverse MPVNR primers: 5'-GCATTGTTTGACCGGCCCCA-3' (nt 814–795). Apart from that, the conditions are similar to those applied in the first amplification.

Respiratory Syncytial Virus (RSV) Detection

The convention PCR method and the primers described by Samransamruajkit *et al*.[62] had been used as the detection method. The reaction mixture contained 2 µl

cDNA, 0.5 µM of RSVABF: 5'-GTCTTACAGCCGTGATTAGG-3' (nt 1672-1691), and RSVABR: 5'-GGGCTTTCTTTGGTTACTTC-3' (nt 2511-2492), 10 µl 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: Initial denaturation at 94ºC for 3 min, followed by 35 amplification cycles consisting of 94°C for 30 sec (denaturation), 55°C for 30 sec (primer annealing), and 72°C for 1 min (extension), and concluded by a final extension step at 72°C for 7 min. The expected products had been shown in 840 bp after 2% agarose gel electrophoresis,

House Keeping Gene Detection

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is the house keeping gene is selected to serve as an internal control for DNA and RNA extraction, using conventional PCR as a detection method. The reaction mixture consisted of 2 µl of DNA or cDNA, 0.5 µM of GAPDHF and GAPHDR for DNA amplification or GAPDHF85 and GAPDHR for cDNA amplification, 10 µl of 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction will be performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: Denaturation at 94 ºC for 3 min, followed by 35 amplification cycles consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, and concluded by a final extension at 72°C for 7 min. The expected result when run into 2% agarose gel electrophoresis and visualize by UV transilluminator had been shown in 200 bp and 497 bp in DNA and cDNA amplification, respectively.

Molecular Characterization and Phylogenetic Analysis of HBoV

All HBoV positive samples were subjected to VP1 gene sequencing. The partial VP1 gene at the 5' end of HBoV was amplified into two segments with two primer sets, VPF1: 5'-GATAACTGACGAGGAAATGCT-3' (nt. 3,009-3,029) and VPR1: 5'-AGTATGTCCATGGAGTTGTGA-3' (nt. 3,731-3,711) for the first segment and VPF2 and VPR2 for the second. The expected product sizes after 2% agarose electrophoresis

were 723 and 648 bp, respectively. PCR conditions had been used as the following complete genome analysis that described below. The PCR products were purified using the Perfectprep Gel Cleanup kit (Eppendorf, Hamburg, Germany). DNA sequencing was performed using the Gene Amp PCR system 9600 (Perkin-Elmer, Boston, MC). The sequencing products were subjected to a Perkin Elmer 310 Sequencer (Perkin-Elmer, Boston, MC) for subsequent sequence analysis. The DNA sequences were analyzed with the BLAST (http://www.ncbi.nlm.gov/BLAST) program and the phylogenetic analyses and genetic comparisons between HBoV strains were performed using the Molecular Evolutionary Genetics Analysis (MEGA) version 4.1 program.

HBoV Complete Coding Sequence Analysis

Two HBoV positive in stool and three HBoV positive samples from NP suction were selected for sequencing the entire coding sequences. The primer set for complete coding sequences amplification was designed based on HBoV complete coding sequence available in the GenBank database as shown in Table 2. All primers were designed for closely matched annealing temperatures so that virtually identical conditions could be applied for the PCR cycle. Briefly, 2 µl of DNA template were added to the reaction mixture containing 10 µl of 2.5X Eppendorf masterMix (Eppendorf, Hamburg, Germany), 0.5 µM of the respective forward and reverse primers and nuclease-free water to a final volume of 25 µl. After 3 min of initial denaturation at 94°C, 35 cycles of amplification (94°C 30 sec, 55°C 30 sec and 72°C 1.30 min) were performed and concluded by a final extension step at 72°C for 7 min. The expected size of the respective PCR product after 2% agarose gel electrophoresis is shown in Table 2. PCR products were purified from the 2% agarose gel using the Perfectprep Gel Cleanup kit (Eppendorf, GmbH, Germany). DNA sequencing was performed using the Gene Amp PCR system 9600 (Perkin-Elmer, Boston, MC). The sequencing products were subjected to a Perkin Elmer 310 Sequencer (Perkin-Elmer, Boston, MC) for subsequent sequence analysis. The sequences were combined with DNA star program and analyzed with BLAST analysis. Then the phylogenic analysis was performed with MEGA 4.1 program.

Phylogenetic Analysis of HBoV2 Partial NS1 Gene

The positive HBoV2 products had been subjected to cycle sequencing reaction. The mixture was making up as the following: 2 µl of 2°PCR product, 1X of BigDye® terminator V3.1 (ABI, Foster City, CA), 5µM of primer, and nuclease-free water to a final volume of 20 µl. The reaction was performed by following condition: 25 cycles of 96°C for 30 sec, 50°C for 20 sec, and 60°C for 4 min. After these the cycle sequencing products were sent to SBS sequencing service, University of Edinburgh, for subsequent sequence analysis. The sequences were combined with Simmonic2005_V1.75 and analyzed with BLAST analysis. Then the phylogenic analysis was performed with MEGA 4.1 program.

Ethical Consideration

All of the protocols of this study in Thailand samples had been approved by Ethics Committee, Faculty of Medicine, Chulalongkorn University. Prior to enrollment, all participating parent gave their written informed consent. And all UK testing samples were approved from Lothian Regional Ethics Committee, UK.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 2 The primers for HBoV complete coding sequences analysis

Name	Direction	Primer sequences (5' to 3')	Position[a]	PCR products (bp)
F5'	F	5'-GCCGGCAGACATATTGGATT-3'	1-20	721
NS1R1	R	5'-GACGTGTAGCCAGAAGAGAT-3'	721-702	
R5'	R	5'-ACCACAAGCGTGGAGCTTTT-3'	243-224	For sequence only
NS1F1	F	5'-GACTAAGCAAGAGGAATGCTA-3'	623-643	838
NS1R2	R	5'-TGACCAACGGCTAGAGGATT-3'	1460-1441	
NS1F2	F	5'-CTTTACAGCTCTCACATATCTT-3'	1347-1368	725
NS1R3	R	5'-GTATCCGTTTTCGTGAAGTGT-3'	2070-2050	
NS1F3	F	5'-CGACTGTACTCTGACAGGAT-3'	1950-1969	807
NP1R2	R	5'-CGAGTAGAGTGCCAGTAGAA-3'	2756-2737	
NP1F2	F	5'-TATCGTCTTGCACTGCTTCG-3'	2591-2610	603
NP1R3	R	5'-AGAGTAGGCGTGATCATGTAA-3'	3193-3173	
VPF1	F	5'-GATAACTGACGAGGAAATGCT-3'	3009-3029	723
VPR1	R	5'-AGTATGTCCATGGAGTTGTGA-3'	3731-3711	
VPF2	F	5'-TTCAGAATGGTCACCTCTACA-3'	3639-3659	648
VPR2	R	5'-CTGTGCTTCCGTTTTGTCTTA-3'	4286-4266	
VPF3	F	5'-AACTTTGACTGTGAATGGGTTA-3'	4172-4193	616
VPR3	R	5'-AAATAGTGCCTGGAGGATGAT-3'	4787-4767	
VPF4	F	5'-ACCAAGGGCTGACAAACACA-3'	4711-4730	589
R3'	R	5'-TGTACAACAACAACACATTAAAAG-3'	5299-5276	
F3'	F	5'-AAAGTGAAGGGTGACTGTAGT-3'	5128-5148	For sequence only

[a] Reference position from GenBank database accession number NC_007455

CHAPTER IV

RESULT

Prevalence of HBoV in Patient with Respiratory Disease

The 302 samples had been collected from admitted patients in one year period (February 2006 to February 2007). The samples were isolated from 173 boys (57.28%) and 129 girls (42.72%) patients who had the age range from 5 days to 14 years.

The study applied PCR and RT-PCR to detect HBoV, other respiratory viruses and GAPDH. Of 302 specimens, 20 (6.62%) were positive for HBoV, (from 14 boys (70%) and 6 girls (30%) patients). All specimens had been collected throughout the year and positive rate of HBoV detection was shown in Fig 5. Among the 302 specimens, we also detected co-infection with other respiratory viruses including RSV in 48 (15.89%) samples, influenza A virus in 33 (10.92%) samples, hMPV in 28 (9.27%) samples, adenovirus in 18 (5.9%) samples, parainfluenza in 14 (4.63%) samples, and influenza B virus in 1 (0.33%) sample. HBoV positive samples co-infected with other respiratory viruses comprised altogether 40% (Table 3). All of viruses mostly detected in the samples collection from 1-3 years old patients (Fig 8).

Prevalence of HBoV in Acute Diarrhea Patient and Healthy People

Of 225 stool specimens collected from children with acute diarrhea, HBoV was detected in 2 samples (0.9%), in which CU8N and CU74W had been collected in September and May, 2006, respectively. Strain CU8N was detected from the girl patient (3 years old) admitted at King Chulalongkorn Memorial Hospital, Bangkok which is the central part of Thailand whereas CU74W was detected from a girl patient (3 months old) admitted at Buriram Provincial Hospital which is located in the north-east of Thailand, both of whom showed acute diarrhea as a symptom without any respiratory diseases. In contrast, HBoV could not be detected in any of the 202 healthy controls (0%, 95%CI = 0-1.5%). There was no statistically significant difference between HBoV cases and the

control group (P = 0.17). The diarrhea stool samples also subjected for rotaviruses screening that published by Theamboonlers A, *et al.* [63]. The result shown that 42.3% were positive human rotavirus A in which one samples had been co-detected with HBoV (CU74W).

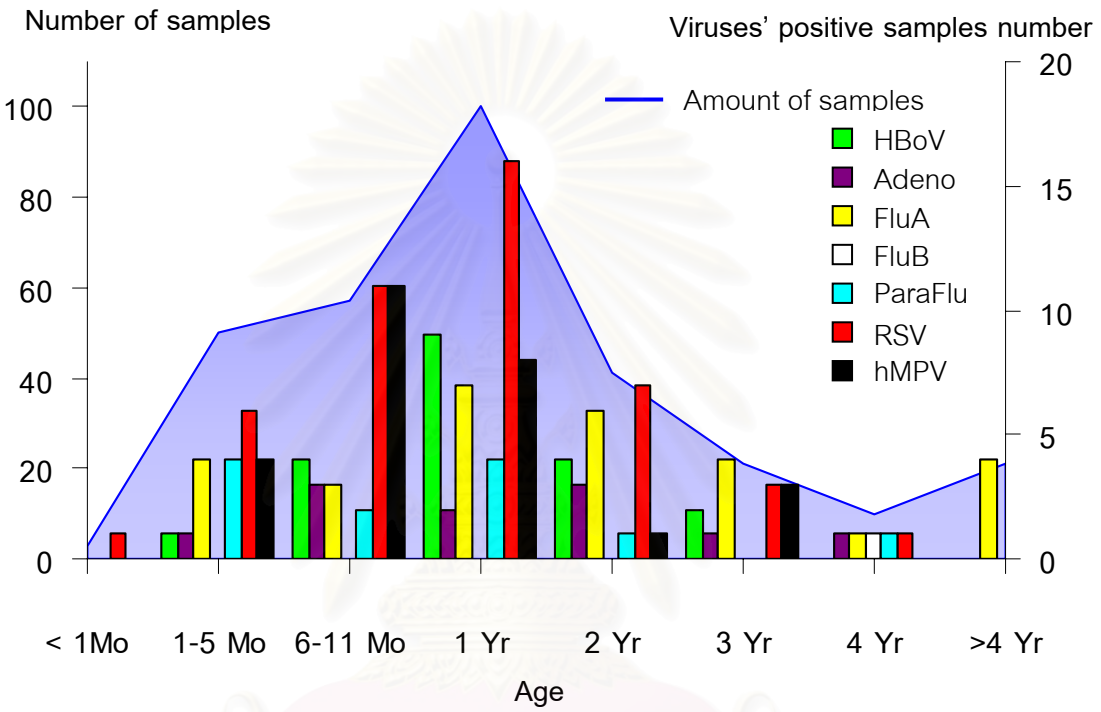


Figure 8. Age groups of studied patient and positivity of virus detection.

Clinical Association of HBoV Infection

The clinical manifestations of HBoV infected patients have been summarized in Table 3. The majority of infants positive for HBoV were boy (14/20), between 4 and 36 months old (median age 12 months). On average, they were hospitalized for 6.15 days (range 2-22 days). The most common clinical symptom in all HBoV infected infants was fever with a mean duration of 3.47 days and other common symptoms such as runny nose (55%) and productive cough (50 %) were found. Generalized rales were the most common (70%) and wheezing the second most common lung signs (35%). Acute bronchiolitis was diagnosed in 9/20 and 11 samples had viral pneumonia. Perihilar

infiltration was ubiquitously present on the chest X-ray of HBoV positive patients (except for CU6 and CU15 where no chest X-ray result was available) (data not shown). Additional diseases of some patients with HBoV infection included congenital heart disease (CU33 and CU74), chronic lung disease (CU 31 and CU171), asthma (CU71) and cow milk protein sensitive enteropathy (CU157).

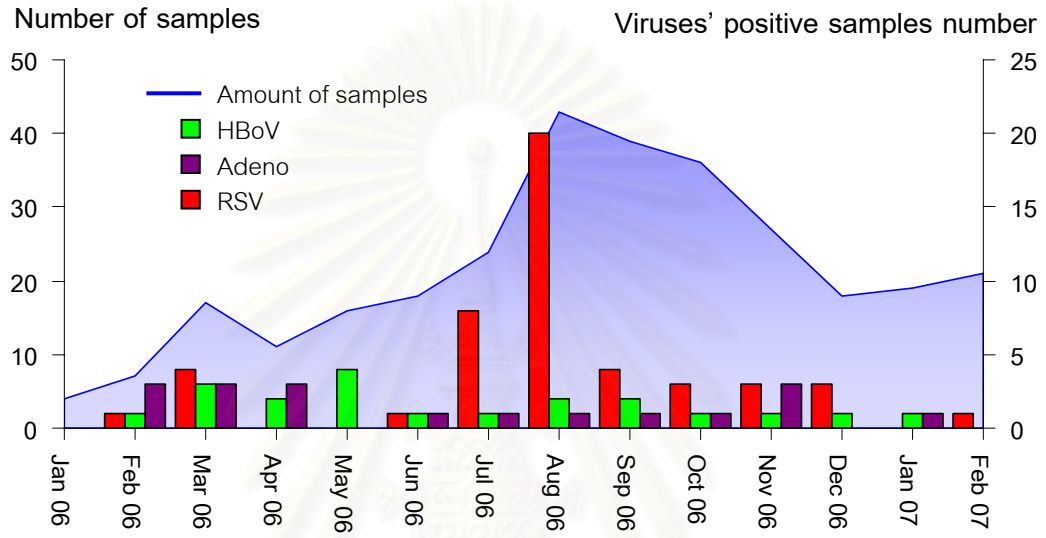


Figure 9. The number detection of HBoV, Adeno, and RSV from February 2006 to February 2007.

Complete Genome Analysis of HBoV

Three HBoV DNA from NP suction designated CU6, CU49, and CU74 and two from stool designated CU8N and CU74W were amplified twice in separate reaction and sequenced both of them to generate the complete coding sequences (GenBank accession numbers EF203920-2 in NP suction strain and EU262978-9 in stool strain, respectively). All five HBoV strains displayed nucleotide lengths of 5,301 base pairs (bp). The NS1, NP1 and capsid proteins VP1 and VP2 comprised 639, 219, 671 and 542 amino acids, respectively.

Table 3 Clinical manifestations in HBoV positive patients

Samples No.	Accession No.	Sex	Age (months)	Duration of hospitalization (Days)	Diagnosis [a]	Clinical manifestations	Viruses detection [b]					
							Adeno	Paraflu	RSV	Flu A	Flu B	hMPV
CU6	EF203920	M	36	2	VP	High fever for 2 days, dry cough, and crepitation both lungs	+	+	+	-	-	-
CU15	EF690641	M	24	4	AB	Low grade fever 4days, clear runny nose, diarrhea, and rhonchi	-	+	-	-	-	-
CU23	EF690642	F	12	2	AB	Fever for 7 days, clear runny nose, wheezing, and rales	-	-	-	+	-	-
CU25	EF690643	M	12	10	AB	Fever for 4 days, intractable cough, diarrhea wheezing, rales, and subcostal retraction	+	-	-	-	-	-
CU31	EF690644	M	7	8	VP	Fever for 7 days, runny nose, expiratory rhonchi, and rales	-	-	-	-	-	-
CU33	EF690645	M	12	7	AB	Fever 5 days, productive cough, breathing difficulty, bilateral rales, and expiratory wheezing	-	-	-	-	-	-
CU40	EF690646	M	4	6	AB	High fever for 1 day, severe productive cough, and expiratory wheezing	-	-	-	-	-	-
CU43	EF690647	M	7	16	AB	Fever for 2 days, clear runny nose, and medium rales	-	-	-	-	-	-
CU49	EF203921	F	24	5	VP	Low fever for 3 days, breathing difficulty, and rales	-	-	-	-	-	-
CU55	EF690648	M	24	4	VP	Fever 2 days, Runny nose, cough, and rales	-	-	-	-	-	-
CU71	EF690649	F	24	5	VP	Fever for 3 days, dry cough, runny nose and crepitation both lungs	-	+	-	-	-	-
CU74	EF203922	M	12	7	VP	Low grade fever, clear runny nose, diarrhea, and medium rales	-	-	-	-	-	-
CU123	EF690650	M	12	3	VP	Fever for 3 days, clear runny nose, and rales	-	-	+	-	-	-
CU157	EF690651	M	11	22	VP	Fever for 7 days, intractable cough, fine crepitations, and hypovolemic shock	-	-	-	-	-	+
CU171	EF690652	F	6	5	VP	Fever for 1 day, runny nose, and rales	-	-	-	-	-	-
CU194	EF690653	M	12	2	VP	Fever 3 days, productive cough, breathing difficulty, bilateral rales, and expiratory wheezing	-	-	-	-	-	-
CU205	EF690654	F	12	2	AB	High fever for 2 days, severe productive cough, and expiratory wheezing	-	-	-	-	-	-
CU218	EF690655	F	36	4	AB	Fever for 2 days, clear runny nose,diarrhea, and medium rales	-	-	+	-	-	+
CU253	EF690656	M	12	4	AB	Low fever for 1 day, breathing difficulty, wheezing, and rales	-	-	-	-	-	-
CU256	EF690657	M	12	5	VP	Fever 7 days, runny nose, diarrhea, and rales	-	-	-	-	-	-

[a] Physician's diagnosis: VP, viral pneumonia; AB, acute bronchiolitis. [b] Other respiratory viruses detected by PCR or RT-PCR.

Adeno, adenovirus; Paraflu, parainfluenza virus; Flu A, influenza A virus; Flu B, influenza B virus; RSV, respiratory syncytial virus and hMPV, human metapneumovirus.

Table 4 Amino acid variation of HBoV detection

Strain	Amino acid changed										
	NS1	NP1		VP1/VP2							
	274	44	47	17	40	72	133	149	149	546	590
ST1[a]	T	S	R	R	L	D	T	A	A	N	T
CU6	-	-	-	-	-	-	-	T	-	-	S
CU49	-	-	-	K	-	N	-	T	-	-	-
CU74	A	-	-	-	S	-	N	T	-	H	S
CU8N	-	N	-	-	S	-	-	T	-	H	S
CU74W	A	-	K	-	S	-	-	-	N	H	S

[a] The reference HBoV prototype NC_007455

The Five HBoV strain nucleotide alignment show many variations along the complete coding sequences that comparison with the ST1 prototype strain, however, those nucleotide substitution could cause a few change at the amino acid residue that summarized in Table 4. Some minor amino acid changes became apparent in the NS1 protein. Due to an A to G substitution at nucleotide position 1,072 an amino acid variation from Thr (T) to Ala (A) was found at position 274 of NP suction strain CU74 and stool strain CU74W. Comparison of the Thai NS1 gene with others based on a sequence identity matrix showed a minimum to maximum identity range of 99.4% to 100%. In the NP1 gene, there NP suction strains have some nucleotide substitutions; however, these substitutions were silent mutations and hence, did not alter the amino acid encoded. Two stool strains had nucleotide substitution that cause the amino acid changed S44N and R47K in CU8N and CU74W, respectively. The sequence identity matrix of the NP1 gene showed a minimum to maximum identity range of 99.2% to 100%. Most variations of nucleotide sequences appeared in the VP1/VP2 gene encoding the capsid protein. The sequence identity matrix of the capsid protein in VP1 specific N-terminal region showed a minimum to maximum identity range of 98.7% to 100% and of 98.2% to 100% for VP2.

Molecular Characterization and Phylogenetic Analysis of HBoV

Phylogenetic analyses and genetic comparisons of HBoV strains were performed using the Molecular Evolutionary Genetics Analysis (MEGA) version 4.1 program. Phylogenetic trees of the complete coding sequence (Fig 10) and each individual gene (Fig 11) of HBoV were constructed.

The results show that HBoV was very conserve in the genome level when compare with the other members of the *Bocavirus* genus (Fig 10A). When the out group had been excluded, however, the different 6 clades of HBoV sequences had been classified. HBoV detected in this study had been classified into clade 1, 3 and 6 (Fig 10B). In all three genes, the NS1 and NP1 genes were highly conserved regions and thus, were not demonstrate differences among HBoV strains (Fig 11A, 11B). As became apparent from the phylogenetic tree of the complete coding sequence, variations of HBoV were shown in VP1 and VP2 , however, the virus could not be divided into clades as same as the appearance in the complete genome phylogenetic tree (Fig 11C).

The constructed phylogenetic trees of the partial VP1 gene (nt. 3,056-4,222) are shown in Figure 9. Alignment of these partial VP1 genes showed minor variations in that the percent identity with the st1 prototype strain ranged from 98.6% to 99.5%. Moreover, this study HBoV strains were mostly related to the Taiwan newly strain (EU984233) (Fig 12).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

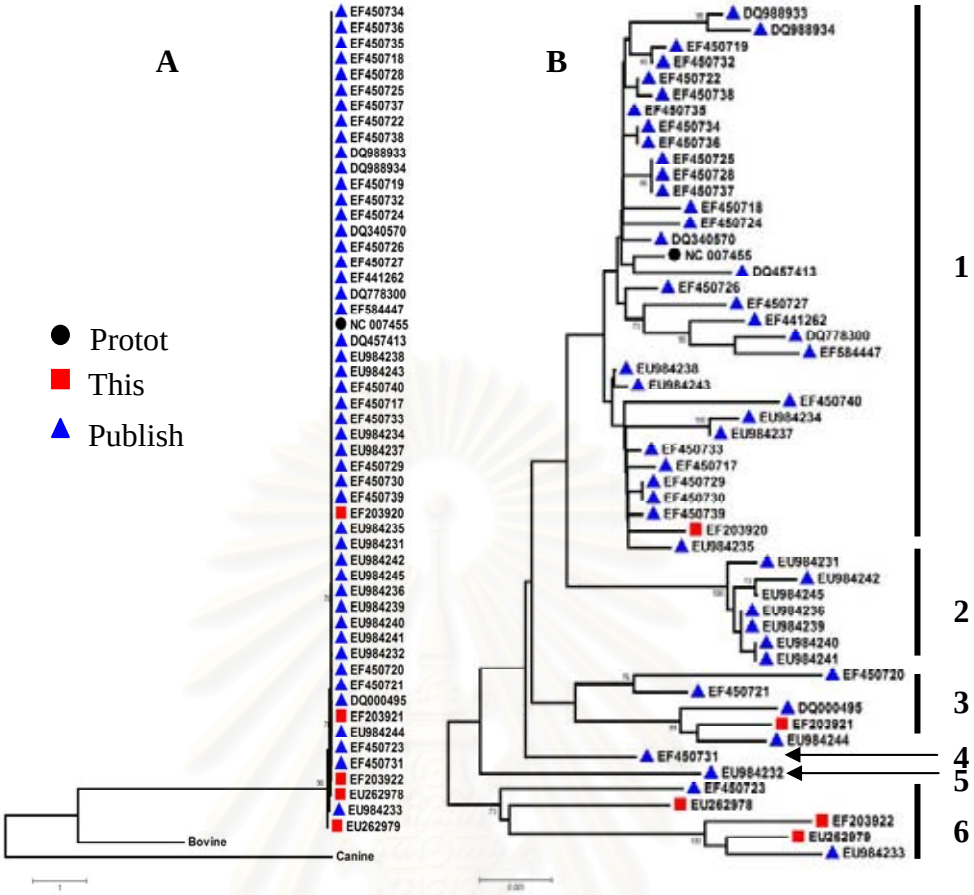


Figure 10. Phylogenetic analysis of the HBoV complete coding sequences in which the other members of *Bocavirus* genus, Bovine parvovirus (BPV) and Canine minute virus (MCV) were served as the out group sequences (A) and the out group were excluded (B).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

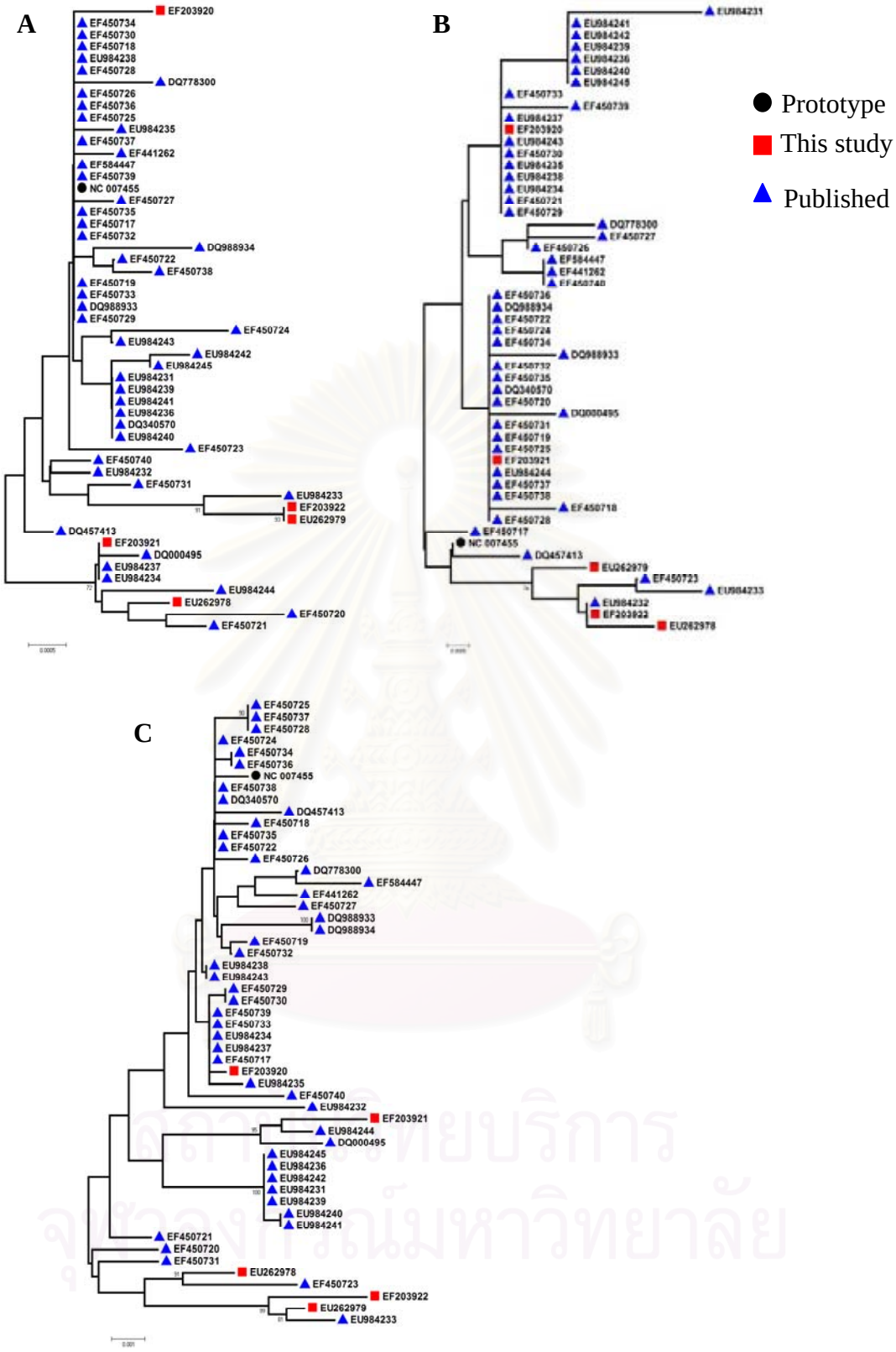


Figure 11. Phylogenetic analyses of, NS1 gene (A), NP1 gene (B), and VP1/VP2 gene (C).

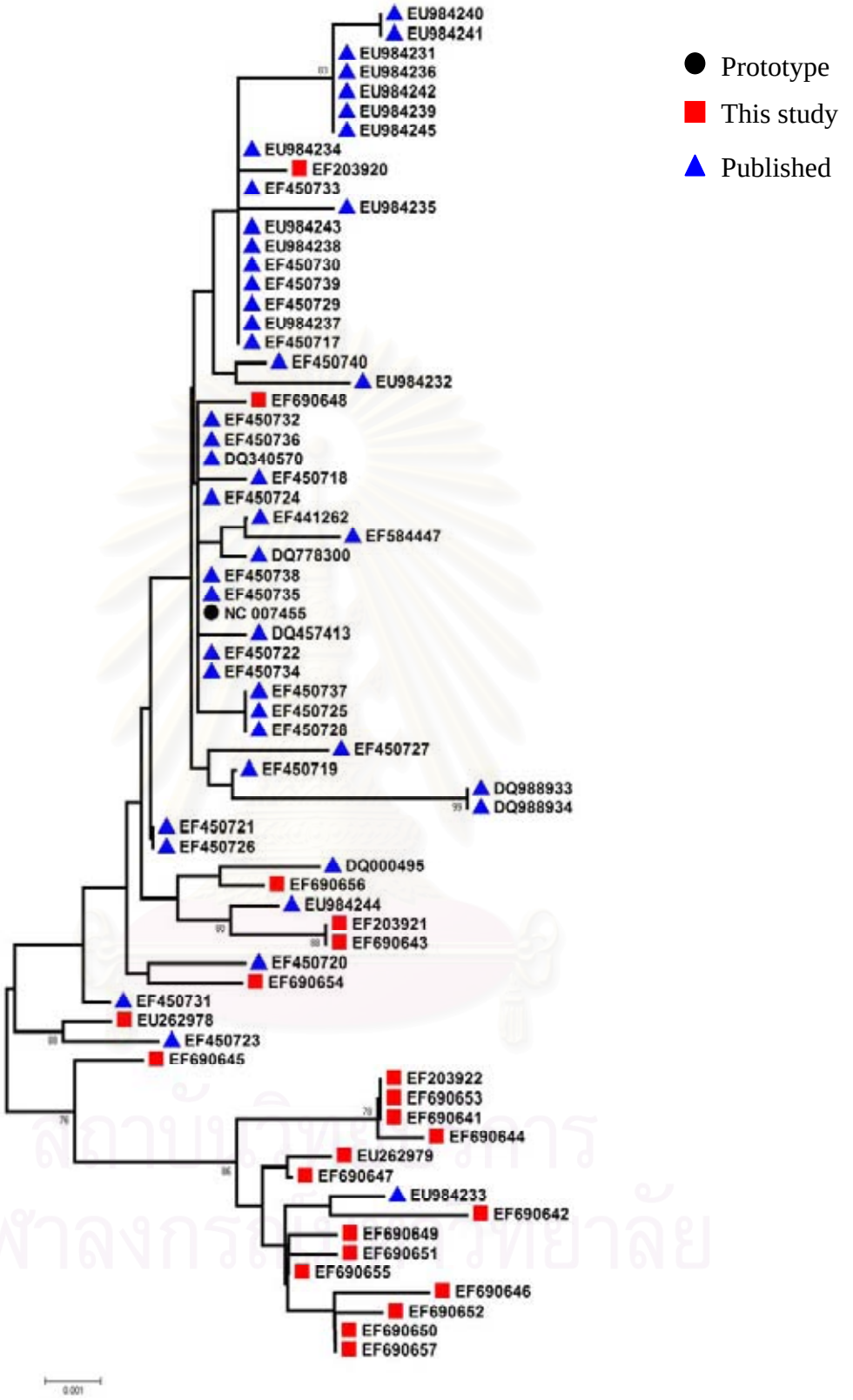


Figure 12. Phylogenetic analysis of HBoV partial VP1 gene (nt. 3,056 -4,222).

HBoV2 Prevalent Study

The study, firstly, was performed in Pakistani children. DNA from stool samples from 57 patients with AFP and from stool from 41 healthy Pakistani children was subjected to amplify the partial NP1 gene of HBoV2. The result showed that 3 samples from children with AFP (including samples from the initial source patient PK5510) and 2 samples from healthy children were positive HBoV2 DNA in which none of these two groups of samples was positive HBoV DNA. Further samples group, respiratory aspirate and stool samples form Edinburgh, UK and NP suction, diarrhea and asymptomatic stool from Thailand, had been used for HBoV2 prevalent study. A total 2 samples (0.61%) from Thailand diarrhea stool samples and 16 samples (0.64%) from UK stool sample were positive HBoV2 DNA. All respiratory aspirates from UK samples, NP suction and asymptomatic stool samples from Thailand showed the negative of HBoV2 DNA detection. The summary of HBoV2 DNA positive samples had been shown in Table 5. The age group of patients that positive HBoV2 generally show in very young (< 2 Yr) and also found in quite old age group (>35 Yr) (Table 5). The diarrhea was shown as the clinical feature in most case of HBoV2 detected patients.

All of HBoV2 positive by using nested PCR were subjected to sequencing analysis and construct phylogenetic tree in which the HBoV and HBoV2 sequences that publish in Genebank database had been compared (HBoV: DQ000495-6, DQ988934, EU262078-9, EF 203920, EF203922, EF450720-1, EF450723, EF450738, EF450740, EF450731, EF584447; HBoV2: FJ170280, PK2255, PK5510). Moreover, the recently sequences analysis from Prof.Eric Delwart, Pacific Blood System Research, University of California, San Francisco, USA also added for reference sequences in this study (TA-210-07, TC-114-06, NI-213, NI-374, NI-327, NI-385). The phylogenetic analysis of partial NS1 gene (nt 1617-2018) had been shown in Fig.13. Five groups of HBoV2 had been classified from the tree (A-E in Fig 13.). Most of samples in this study belonging to group A, but 2 of them (TH-SR47 and UK-2244) had been classified as group B. Interestingly, UK-2139 had been classified as group E that recently define as the recombinant between HBoV and HBoV2 (unpublished data). The nucleotide percent identity of group A-D show around 95.0-97.0% and 81.3-82.8% when compare with HBoV2 and HBoV,

respectively. Whereas in group E show 83.8-84.6% and 90.5-91.0% as nucleotide percent identity when compared with HBoV2 and HBoV, respectively.

Table 5 The summarize of HBoV2 positive samples

Sample	Age group	Sex	Clinical detail
UK-452[a]	6-12 Mo	Male	No data
UK-648[a]	0-3 Mo	Female	Diarrhea
UK-649[a]	>65 Yr	Male	No data
UK-1555[a]	1-2 Yr	Female	Diarrhea
UK-1557[a]	1-2 Yr	Female	Diarrhea
UK-1906	11-15 Yr	Male	No data
UK-1933[a]	6-12 Mo	Male	Diarrhea
UK-2139	1-2 Yr	Male	No data
UK-2244[a]	6-12 Mo	Male	No data
UK-2289[a]	36-65 Yr	Female	Diarrhea
UK-2343[a]	36-65 Yr	Female	Diarrhea
UK-2387[a]	3-5 Yr	Male	Diarrhea
UK-2390[a]	>65 Yr	Female	No data
UK-2451[a]	1-2 Yr	Female	Diarrhea and vomiting
UK-2454[a]	>65 Yr	Female	No data
UK-2480[a]	6-12 Yr	Male	No data
TH-SR47[b]	3-5 Yr	Male	No data
TH-SR54[b]	3-5 Yr	Male	No data

[a]: samples from UK, [b]: samples from Thailand, Yr: year, Mo: month.

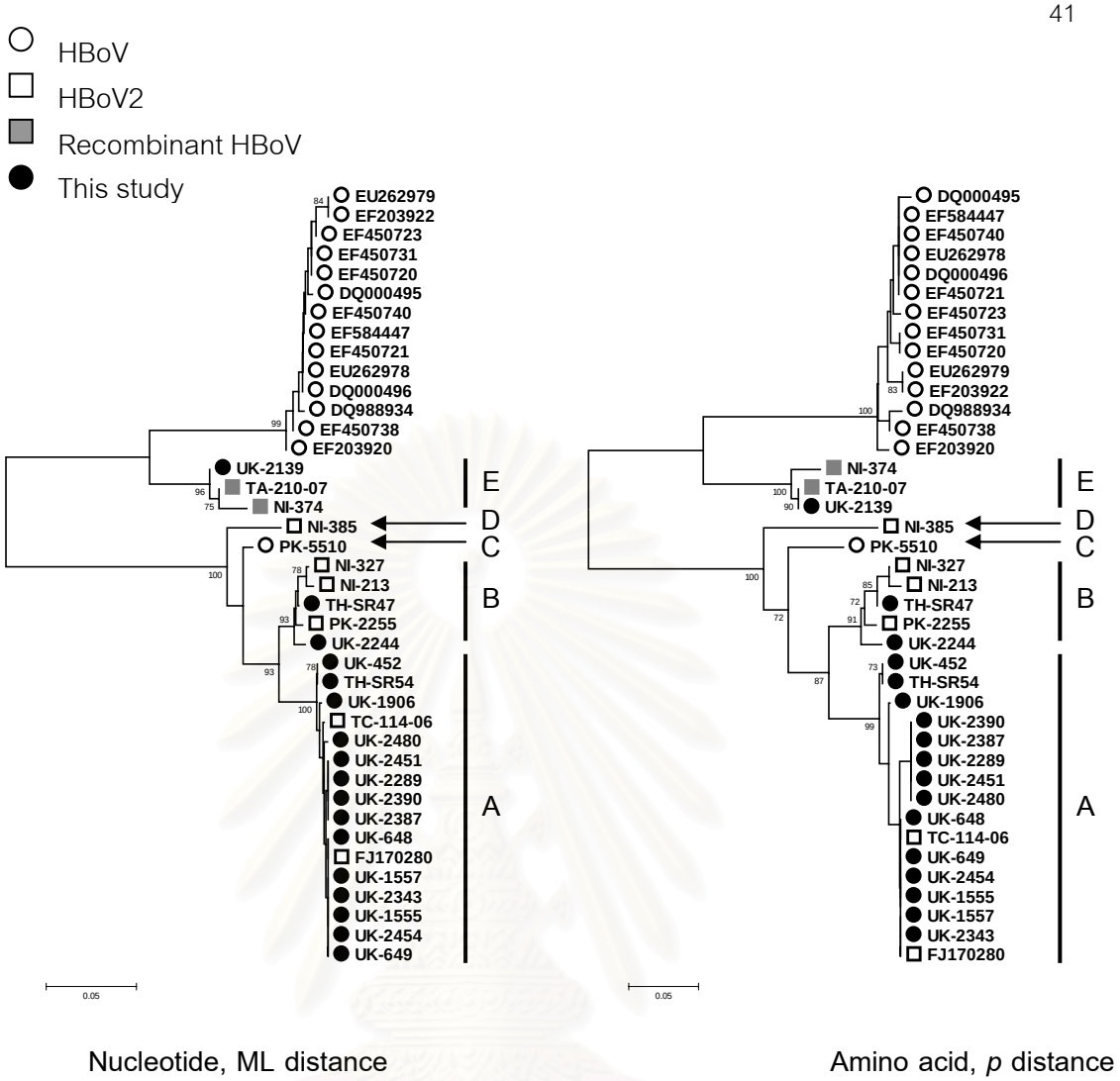


Figure 13. Phylogenetic tree of partial NS1 gene (nt 1617-2018) of HBoV and HBoV2

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER V

DISCUSSION AND CONCLUSION

DISCUSSION

Epidemiology Study and Clinical Association with HBoV and HBoV2

In the past few years, the metagenomic that applying for molecular techniques had led to the discovery of HBoV as a novel virus in *Parvoviridea* family, *Bocavirus* genus which is apparently associated with respiratory tract illness in humans [1]. Due to lack of suitable culture systems and animal models to propagate the virus previous studies could not meet with Koch's postulates in order to clarify that whether HBoV can cause respiratory illness. However, recently discovery of many causative viruses base on the molecular techniques, Fredricks and Relman had been proposed the alternative guidelines for establishing microbial disease causation in the absence of a cultivated or purified microorganism [57]. The epidemiological study is the one of data for conclude in those criteria. Since HBoV was identified, the global prevalence study had been reported with a different incidence rate (1.5% to 19% that show in Table 1), depended on the inclusion criteria and the types of samples collection.

In this study, the NP suctions had been collected from pediatric patients hospitalized with acute lower respiratory tract illness. HBoV has been detected in 20 of 302 NP suction samples (6.62%) that closed to the study in Hong Kong and Spain (Table 1.). Asymptomatic control groups were not included in this study; however, the study in USA had been investigated in well-children which serve as the control group. The results showed that the HBoV was detected in 5.2% symptomatic group [25]. Another study at the rural area of Thailand had been reported a slightly lower incident rate (4.5%) when compare with our study. The study, outpatients with no respiratory infection symptoms as the control group in which HBoV DNA can be detected in 3 of 280 samples (1%) [18]. The other common respiratory infection viruses including influenza A, influenza B, parainfluenza, RSA, adenovirus, and hMPV also detected in our

study. The HBoV co-infection with other respiratory viruses was found in 40% of the NP suction samples tested. RSV and parainfluenza virus were frequently detected as co-infecting viruses. However, some of the respiratory viruses were not included in this study, for examples; rhinoviruses and coronaviruses. Therefore, the percentage of co-infection may be higher than this report. The high rate co-infection of HBoV and other respiratory viruses also had been reported from many epidemiological studies [1, 3, 4, 6-34, 39-42]. The replication of parvoviruses mostly depends on the host cellular function, for examples, parvovirus B19, *Erythovirus* genus, only replicates during the DNA replication phase of host cell or adeno-associated virus in *Dependovirus* genus that can replicate in the host cell that induced the DNA replication by co-infected adenovirus or helper viruses. However, the mostly co-infection viruses in this study had not the strategy to induce the host replication. It may be like the other member of viruses in the same genus, BPV and CMV, which do not require the helper viruses to facilitate its own replication [64].

The seasonal detection of HBoV can not be demonstrated in this study in which the few [1-3] HBoV-positive samples found in each month around the year (Fig 9). Many studies reported the seasonal detection of HBoV [4, 12, 13, 16, 17, 25], however, when taken the result together, it is no regular seasonal occurrence. Whereas the other three viruses that show the most incident rate in this study; RSV (Fig 9), Flu A (data not shown), and hMPV (data not shown), were shown the seasonal distribution that peaked in July to September in which correlated to the number of samples collection. Moreover, the study of Mannig A *et al*. reported a similarity in seasonal appearance between HBoV and RSV [12].

The clinical features commonly found in HBoV positive patients were fever and productive cough. Bilateral rales and wheezing were among the most common abnormal lung signs observed and almost equally found in these patients (Table 1). These findings might indicate both lung parenchyma and airway involvement by this pathogen. Furthermore, the detection of this virus in specimens aspirated from the nasopharynx together with the significant lower respiratory tract illness indicated the lung pathology in these patients.

Many studies focusing in GI secretion since HBoV had been detected in stool samples [27-30]. The stool from children with diarrhea and asymptomatic had been included in this study. The results show that HBoV could only be found in the stool of two children with acute gastroenteritis symptoms but could not be detected in the asymptomatic control samples. The prevalence in this study was lower than that published in Spain; 9.1% [27], but closed to the results obtained in South Korea; 0.8% [28], Brazil; 2% [29] and Hong Kong; 2.1% [30] (Table 1). The HBoV also found in very young patient that similar to the prevalence in respiratory screening. Unfortunately, in this study HBoV analysis was not performed on isolates obtained from respiratory secretions and thus, it can not be excluded that HBoV detected in patients had not originally infected the respiratory tract and subsequently been passed through the GI tract. However, previous studies have demonstrated HBoV isolation from patients diagnosed with gastroenteritis only. [27-30].The study presented here has had its limitations due to the small population size and insufficient amounts of specimens to test for other pathogens except HBoV and rotavirus. The screening in all asymptomatic children stools which serve as controls show negative of HBoV DNA. And when comparing with diarrhea samples do not show significant difference in statistical testing. Moreover the small sample sizes collection in both case and control group and a short period of time for control group collection must be concerned. Therefore, it should be over to conclude that this virus can establish the gastroenteritis from this study. Hence, a large number of cases and controls ought to be evaluated. These preliminary data may prove crucial to advance our understanding of HBoV infection of the GI tract. However, the pathogenicity of this virus in the respiratory and GI tract remains to be elucidated.

After HBoV had been discoveried and many studies of this virus reported the global isolation, the new species that share around 73.8-74.5% identity in whole genome nucleic acid of HBoV had been identified from stool samples and was named as HBoV2. The published primer sequences for HBoV detection all contain several mismatches with HBoV2 sequences that would likely not amplified HBoV2 DNA. Therefore the recently study likely to report the HBoV prevalence only. Thus, HBoV2 may represent the

additional undetected etiological agent in enteric or respiratory disease. NP aspirates and stool samples that collected from Thailand and Edinburgh, UK had been included in this study. Interestingly, all respiratory aspirates in both Thai and UK patient show negative result of HBoV2 screening but only found in diarrhea stool samples, with incidence rate, 0.61% and 0.64%, respectively. This result may suggest a difference tissue tropism that may influence its transmission route and ability to infect systemically and establish persistence. However, the HBoV2 can be detected in very young age and do not have any significant difference between male and female as HBoV detection.

Molecular Characterization and Phylogenetic Analysis of the HBoV and HBoV2

The complete coding sequences of HBoV in Thailand samples were determined with nine PCR products amplified by using overlapping primer sets and subjected to sequencing. Upon analysis, 5 HBoV positive samples, 3 from NP suction and 2 from diarrhea stool samples, sequences were submitted to the GenBank database. All sequences were more than 97% identical to the HBoV sequences previously published in GenBank database. Together with the phylogenetic tree analysis when comparing with other member of the same genus, this virus is very conserve in nucleotide and amino acid level. However, when the outer group sequences were excluded, these viruses showed little variation, in which almost uniformly appeared in the capsid protein (VP1 and VP2) that can be identified as 6 difference clades (Fig 10). NS1 and NP1, whereas, represent the most conserved regions (Fig 11). The HBoV sequences do not significantly relate to specific clade in phylogenetic tree study and concurrently with the prevalence result at the recently study that shown in Table 1 indicated that this virus could be global distributed and did not restrict in the specific region.

The newly discovered species of HBoV2 had been shown higher variations in complete coding nucleotide sequences, about 74% identity, when compared with HBoV. HBoV2 showed the variation at NP1 and NS1, in contrast with HBoV that was shown in capsid VP1/VP2 gene (Fig 14.). At the first investigation, this newly HBoV2 could be classified as 3 groups based on their complete coding sequences in

phylogenetic tree analysis. It's likely the recombinant among these 3 groups of HBoV2 had been observed (Fig.12). Moreover, the recombinant between HBoV and HBoV2 also clearly investigated in this study. UK2134 was classified as the recombinant strain as same as the unpublished data from USA. (TA-210-07 and NI-374) that show in Fig.13 in which had been shown 84.3% and 90.8% as the percent identity when comparing with PK-5510 (HBoV2) and st2 (DQ000496,HBoV), respectively. From this seem to be that HBoV2 had more variation than HBoV. However, the large number of samples screening should be further studies to clarify this hypothesis.

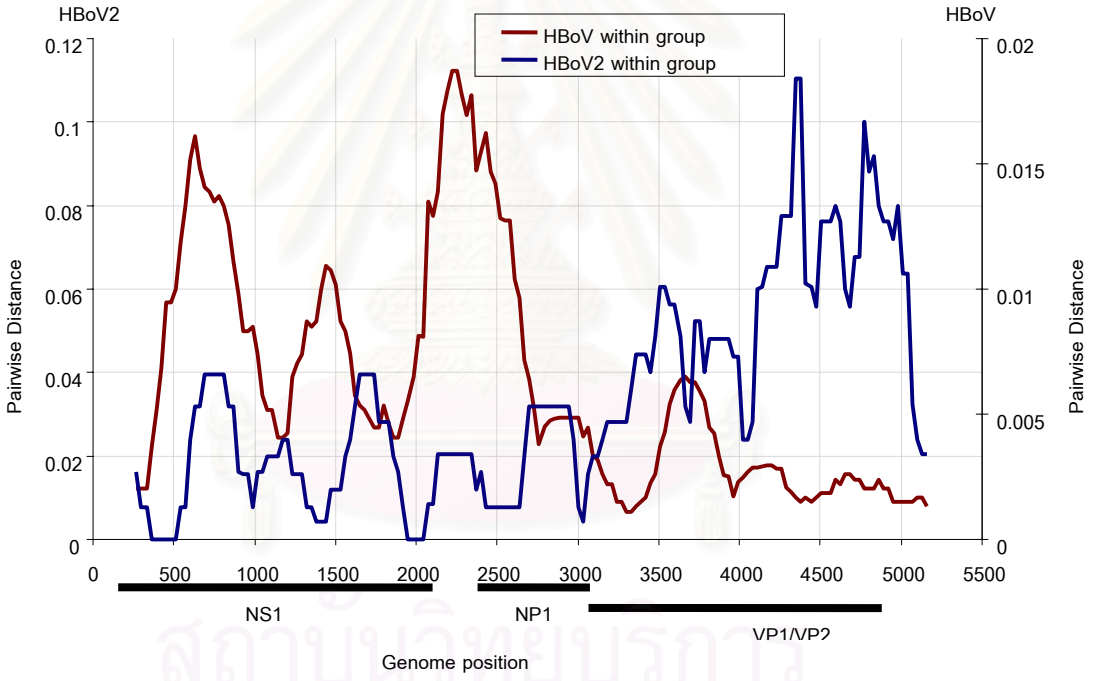


Figure 14. The sliding window analysis of HBoV and HBoV2. Divergence within group of nearly complete nucleotide genome sequences of HBoV and HBoV2. Sequences were aligned to determine sequence divergence (uncorrected *p* distances), by use of a window size of 300 and 30 base increments.

CONCLUSION

Two different of sample types, large number samples size, and different geographic samples collection had been incorporated for HBoV screening in this study. From this, we detected HBoV infection in 6.62% and co-infection with other respiratory viruses in 40% of the NP suctions obtained from infants and children with acute lower respiratory tract illness with non-specific clinical features and age distribution. Seasonal appearance of HBoV was not significant. Based on phylogenetic analysis of the VP1 gene, the low genetic diversity was defined. The complete coding sequences of HBoV indicate that although HBoV is a conserved virus, the sequence analysis showed that amino acid variations almost uniformly appear in the capsid protein (VP1 and VP2). NS1 and NP1 represent the most conserved regions, which should be utilized for HBoV detection. HBoV could only be found in the stool of two children with acute gastroenteritis symptoms but could not be detected in the control samples. However, there is no significant different between these two group of samples screening (p=0.17).

The newly species of parvovirus had been identified, “HBoV2”, that shown about 74% identity to HBoV. The study indicated that HBoV2 has been widely in separated areas (UK, Thailand and Pakistan) and likely global distribution. The absence of HBoV2 isolated from respiratory samples may suggest the different tissue tropism with HBoV that may influence its transmission route and ability to infection. However, HBoV2 show the similar targeting of young children, with most occurring below the age of one year. At the genome level, HBoV2 show more variation than HBoV in which the recombination within HBoV2 species and between HBoV also be investigated from this study. Base on the limitation of the absent of cultivation system and the Koch’s postulates can not be fulfill, the epidemiological and molecular study comparing the presentation of clinical manifestation of the viruses should be provided the more understanding of etiological induction of these viruses.

REFERENCES

[1] Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc. Natl. Acad. Sci. U S A. 102 (2005): 12891-12896.

[2] Chieochansin T, Chutinimitkul S, Payungporn S, Hiranras T, Samransamruajkit R, Theamboolers A, Poovorawan Y. Complete coding sequences and phylogenetic analysis of Human Bocavirus (HBoV). Virus Res. 129 (2007): 54-55.

[3] Arnold JC, Singh KK, Spector SA, Sawyer MH. Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin Infect Dis. 1 (2006): 283-288.

[4] Bastien N, Chui N, Robinson JL, Lee BE, Dust K, Hart L, Li Y. Detection of human bocavirus in Canadian children in a 1-year study. J Clin Microbiol. 45 (2007): 610-613.

[5] Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infection. J Med Virol. 78 (2006): 1232-1240.

[6] Bastien N, Brandt K, Dust K, Ward D, Li Y. Human bocavirus infection, Canada. Emerg Infect Dis. 12 (2006): 848-850.

[7] Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, et al. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000-2005. Clin Infect Dis. 43 (2006): 585-592.

[8] Chung JY, Han TH, Kim CK, Kim SW. Bocavirus infection in hospitalized children, South Korea. Emerg Infect Dis. 12 (2006): 1254-1256.

[9] Foulongne V, Olejnik Y, Perez V, Elaerts S, Rodiere M, Segondy M. Human bocavirus in French children. Emerg Infect Dis. 12 (2006): 1251-1253.

[10] Kaplan NM, Dove W, Abu-Zeid AF, Shamoon HE, Abd-Eldayem SA, Hart CA. Human bocavirus infection among children, Jordan. Emerg Infect Dis. 12 (2006): 1418-1419.

[11] Ma X, Endo R, Ebihara T, Ishiko H, Ariga T, Kikuta H. Detection of human bocavirus in Japanese children with lower respiratory tract infection. J Clin Microbiol. 44 (2006): 1132-1134.

[12] Manning A, Russell V, Eastick K, leadbetter H, Templeton K, Simmon P. Epidermiology profile and clinical associations of human bocavirus and other human parvovirus. J Infect Dis. 194 (2006): 1283-1290.

[13] Neske F, Blessing K, Tollmann F, Schubert J, Rethwilm A, Kreth HW, et al. Real-Time PCR assays for detection of bocavirus in human specimens. J Clin Microbiol. 44 (2006): 3231-3135.

[14] Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol. 35 (2005): 99-102.

[15] Smuts H, Hardie D. Human bocavirus in hospitalized children, South Africa. Emerg Infect Dis. 12 (2006): 1457-1458.

[16] Weissbrich B, Neske F, Schubert J, Tollmann F, Blath K, Blessing K, et al. Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect Dis. 6 (2006): 109.

[17] Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, et al. Human bocavirus and acute wheezing in children. Clin Infect Dis. 44 (2007): 904-910.

[18] Fry AM, Lu X, Chittaganoitch M, Peret T, Fischer J, Dowell SF, et al. Human bocavirus: A novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis. 195 (2007): 1038-1045.

[19] Kleines M, Scheithauer S, Rackowitz A, Ritter K, Hausler M. High prevalence of human bocavirus detected in young children with severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel Real-Time PCR protocol. J Clin Microbiol. 45 (2007): 1032-1034.

[20] Maggi F, Andreoli, Pifferi M, Meschi S, Rocchi J, Bendinelli M. Human bocavirus in Italian patients with respiratory diseases. J Clin Virol. 38 (2007): 321-328.

[21] Monteny M, Niesters HGM, Moll HA, Berger MY. Human bocavirus in febrile children, the Netherlands. Emerg Infect Dis.13 (2007): 180-182.

[22] Naghipour M, Cuevas LE, Bakhshinejad T, Dove W, Hart CA. Human bocavirus in Iranian children with acute respiratory infections. J Med Virol. 79 (2007): 539-543.

[23] Qu XW, Duan ZJ, Qi ZY, Xie ZP, Gao HC, Liu WP, et al. Human bocavirus infection, people's republic of China. Emerg Infect Dis. 13 (2007): 165-168.

[24] Schenk T, Huck B, Forster J, Berner R, Neumann-Haefelin D, Falcone V. Human bocavirus DNA detected by quantitative real-time PCR in two children hospitalized for lower respiratory tract infection. Eur J Clin Microbiol Infect Dis. 26 (2007): 147-149.

[25] Kesebir D, Vazquez M, Weibel C, Shapiro ED, Ferguson D, Landry ML, et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis. 194 (2006): 1276-1282

[26] Simon A, Groneck P, Kupfer B, Kaiser R, Plum G, Tillmann RL, et al. Detection of bocavirus DNA in nasopharyngeal aspirates of a child with bronchiolitis. J Infect. 54 (2007): e125-127.

[27] Vicente D, Cilla G, Montes M, Pérez-Yarza EG, Pérez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis. 13 (2007): 636-637.

[28] Lee JI, Chung JY, Han TH, Song MO, Hwang ES. Detection of human bocavirus in children hospitalized because of acute gastroenteritis. J Infect Dis. 196 (2007): 994-997.

[29] Albuquerque MC, Rocha LN, Benati FJ, Soares CC, Maranhão AG, Ramírez ML, et al. Human bocavirus infection in children with gastroenteritis, Brazil. Emerg Infect Dis. 13 (2007): 1756-1758.

[30] Lau SK, Yip CC, Que TL, Lee RA, Au-Yeung RK, Zhou B et al. Clinical and molecular epidemiology of human bocavirus in respiratory and fecal samples from children in Hong Kong. J Infect Dis. 196 (2007): 986-993.

[31] Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet. 367 (2006): 1747-1757.

[32] van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med. 7 (2001): 719-724.

[33] van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, et al. Identification of a new human coronavirus. Nat Med. 10 (2004): 368-373.

[34] Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, Wong BH, Poon RW, et al. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol. 79 (2005): 884-895.

[35] Qu XW, Liu WP, Qi ZY, Duan ZJ, Zheng LS, Kuang ZZ, *et al*. Phospholipase A2-like activity of human bocavirus VP1 unique region. Biochem Biophys Res Commun. 365 (2008): 158-163.

[36] Brieu N, Gay B, Segondy M, Foulongne V. Electron microscopy observation of human bocavirus (HBoV) in nasopharyngeal samples from HBoV-infected children. J Clin Microbiol. 45 (2007): 3419-3420.

[37] Kahn JS, Kesebir D, Cotmore SF, D'Abramo A Jr, Cosby C, Weibel C, et al. Seroepidemiology of human bocavirus defined using recombinant virus-like particles. J Infect Dis. 198 (2008): 41-50.

[38] Endo R, Ishiguro N, Kikuta H, Teramoto S, Shirkoohi R, Ma X, et al. Seroepidemiology of human bocavirus in Hokkaido prefecture, Japan. J Clin Microbiol. 45 (2007): 3218-3223.

[39] Lin JH, Chiu SC, Lin YC, Chen HL, Lin KH, Shan KH, et al. Clinical and genetic analysis of Human Bocavirus in children with lower respiratory tract infection in Taiwan. J Clin Virol. 44 (2009): 219-224.

[40] Villa L, Melón S, Suárez S, Alvarez-Argüelles ME, Gónzalez D, Morilla A, et al. Detection of human bocavirus in Asturias, Northern Spain. Eur J Clin Microbiol Infect Dis. 27 (2008): 237-239.

[41] Chung JY, Han TH, Kim SW, Kim CK, Hwang ES. Detection of viruses identified recently in children with acute wheezing. Med Virol. 79 (2007):1238-1243.

[42] Chow BD, Huang YT, Esper FP. Evidence of human bocavirus circulating in children and adults, Cleveland, Ohio. J Clin Virol. 43 (2008): 302-306.

[43] Catalano-Pons C, Giraud C, Rozenberg F, Meritet JF, Lebon P, Gendrel D. Detection of human bocavirus in children with Kawasaki disease. Clin Microbiol Infect. 13 (2007): 1220-1222.

[44] Pozo F, García-García ML, Calvo C, Cuesta I, Pérez-Breña P, Casas I. High incidence of human bocavirus infection in children in Spain. J Clin Virol. 40 (2007): 224-228.

[45] Hindiyeh MY, Keller N, Mandelboim M, Ram D, Rubinov J, Regev L, et al. High rate of human bocavirus and adenovirus coinfection in hospitalized Israeli children. J Clin Microbiol. 46 (2008): 334-337.

[46] Li ZL, Tang LY, Xie ZD, Tan XJ, Li CS, Cui AL, et al. Human bocavirus in children suffering from acute lower respiratory tract infection in Beijing Children's Hospital. Chin Med J (Engl). 121 (2008): 1607-1610.

[47] Foulongne V, Rodière M, Segondy M. Human Bocavirus in children. Emerg Infect Dis. 12 (2006): 862-823.

[48] Redshaw N, Wood C, Rich F, Grimwood K, Kirman JR. Human bocavirus in infants, New Zealand. Emerg Infect Dis. 13 (2007): 1797-1799.

[49] Longtin J, Bastien M, Gilca R, Leblanc E, de Serres G, Bergeron MG, et al. Human bocavirus infections in hospitalized children and adults. Emerg Infect Dis. 14 (2008): 217-221.

[50] Jacques J, Moret H, Renois F, Lévêque N, Motte J, Andréoletti L. Human Bocavirus quantitative DNA detection in French children hospitalized for acute bronchiolitis. J Clin Virol. 43 (2008): 142-147.

[51] Völz S, Schildgen O, Klinkenberg D, Ditt V, Müller A, Tillmann RL, et al. Prospective study of Human Bocavirus (HBoV) infection in a pediatric university hospital in Germany 2005/2006. J Clin Virol. 40 (2007): 229-235.

[52] Smuts H, Workman L, Zar HJ. Role of human metapneumovirus, human coronavirus NL63 and human bocavirus in infants and young children with acute wheezing. J Med Virol. 80 (2008): 906-912.

[53] Tan BH, Lim EA, Seah SG, Loo LH, Tee NW, Lin RT, et al. The incidence of human bocavirus infection among children admitted to hospital in Singapore. J Med Virol. 81 (2009): 82-89.

[54] Cheng WX, Jin Y, Duan ZJ, Xu ZQ, Qi HM, Zhang Q. et al. Human bocavirus in children hospitalized for acute gastroenteritis: a case-control study. Clin Infect Dis. 47 (2008): 161-167.

[55] Yu JM, Li DD, Xu ZQ, Cheng WX, Zhang Q, Li HY, et al. Human bocavirus infection in children hospitalized with acute gastroenteritis in China. J Clin Virol. 42 (2008): 280-285.

[56] Campe H, Hartberger C, Sing A. Role of Human Bocavirus infections in outbreaks of gastroenteritis. J Clin Virol. 43 (2008): 340-342.

[57] Fredericks DN and Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clin Microbiol Rev. 9 (1996): 18-33.

[58] Kapoor A, Victoria J, Simmonds P, Wang C, Shafer RW, Nims R, Nielsen O,Delwart E. A highly divergent picornavirus in a marine mammal. J Virol. 82 (2008): 311-320.

[59] Krafft AE, Russell KL, Hawksworth AW, McCall S, Irvine M, Daum LT, et al. Evaluation of PCR testing of ethanol-fixed nasal swab specimens as an augmented surveillance strategy for influenza virus and adenovirus identification. J Clin Microbiol. 43 (2005): 1768-1775.

[60] Chi XS, Hu A, Bolar TV, Al-Rimawi W, Zhao P, Tam JS, et al. Detection and characterization of new influenza B virus variants in 2002. J Clin Microbiol. 43 (2005): 2345-2349.

[61] Thanasugarn W, Samransamruajkit R, Vanapongtipagorn P, Prapphal N, Van den Hoogen B, Osterhaus AD, et al. Human metapneumovirus infection in Thai children. Scand J Infect Dis. 35 (2003): 754-756.

[62] Samransamruajkit R, Moonviriyakit K, Vanapongtipagorn P, Prapphal N, Deerojanawong J, Poovorawan Y. Plasma endothelin-1 in infants and young children with acute bronchiolitis and viral pneumonia. Asian Pac J Allergy Immunol 20 (2002): 229-234.

[63] Theamboonlers A, Bhattarakosol P, Chongsrisawat V, Sungkapalee T,Wutthirattanakowit N, Poovorawan Y. Molecular characterization of group A human rotaviruses in Bangkok and Buriram, Thailand during 2004-2006 reveals

the predominance of G1P[8], G9P[8] and a rare G3P[19] strain. Virus Genes. 36 (2008): 289-298.

[64] Knipe DM, and Howley PM. Chapter 69. Fields Virology. 4th edtion. Philadelphia : Lippincott Williams&Wlikins, (2001): 2327-2359.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX

Materials and Laboratory Equipment

Sample collection

- Bovine Serum Albumin (BSA) (GIBCO)
- Penicillin-Streptomycin (10,000 units/ml) (GIBCO)
- Phosphate Buffer Saline (PBS Tablets) (Bio Basic Inc.)

DNA and RNA extraction

- TRI REAGENT ® LS (Molecular Research Center)
- Sodium hydroxide (MERCK)
- Diethyl pyrocarbonant (DEPC)
- MiniElute Virus Spin Kit (Qiagen)

Reverse transcription

- M-MLV Reverse Transcriptase (Promega)
- AMV Reverse Transcriptase (Promega)
- RNasin Ribonuclease Inhibitor (Promega)
- Set of dATP, dCTP, dGTP, dTTP (Promega)
- Random Primer (Promega)
- Diethyl pyrocarbonant (DEPC)

สำนักวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Polymerase chain reaction

- Eppendrof MasterMiX (2.5X) (Eppendrof)
- AccessQuickTM Master Mix (Promega)
- SYBR Green I (Applied Biosystems)
- GoTaq™ (Promega)
- 10X GoTaq™ reagent buffer (Promega)
- Set of dATP, dCTP, dGTP, dTTP (Promega)

Electrophoresis and DNA staining

- Gene Ruler 100 bp DNA Ladder Plus (Fermentas)
- Seakem LE agarose (BioWhittaker Molecular Application)
- Ethidium Bromide (SIGMA)

Nucleotide Sequencing

- Perfectprep Gel Cleanup (Eppendorf)
- ABI PRISM Bigdye Termibator v3.1 Cycle Sequencing Kit (Applied Biosystems)
- 310 Genetic Analyzer Performance Optimized Polymer 6 (Applied Biosystems)
- Buffer (10X) with EDTA (Applied Bisystems)
- Template suppression Reagent (TSR) (Applied Biosystems)

General Materials for Bimolecular Research

- MicroAmp PCR tube (Perkin Elemer)
- Microcentrifuge tube : 0.5 and 1.5 µl (AxyGen® Scientific)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

- Polypropylene conical tube : 15 and 50 ml (AxyGen® Scientific)
- Pipette tip : 10 µl, 200 µl, and 1,000 µl (AxyGen® Scientific)

Equipment

- Centrifuge
- Refrigerated microcentrifuge
- -20°C freezer
- -70°C freezer
- DNA Thermal Cycler 9600 (Perkin Elmer)
- Mastercycler personal (Eppendorf)
- Rotor-Gene RG-3000 (Corbett Research)
- Gel Doc 1000 UV transilluminator (Biorad)
- Bio photometer (Eppendorf)
- Class II Microbiological Safety Cabinent (Envair)
- PCR Cabinet (Augusta)
- Perkin-Elmer 310 Sequencer (PE Applied Biosystems)
- Autoclave (Hydroclave MC10 Harvey)
- Hot air oven (Memmert)
- Multi-block heater (Lab-line)
- Microwave oven

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Software for bioinformatics analysis

- CLUSTAL X program (version 1.8)
- OLIGOS primer design software (version 9.1)
- BioEdit Sequence Aligment Editor (version 5.0.9)
- Chromas Lite (version 2.0.1)
- Molecular Evolution Genetics Analysis (MEGA) (version 4.1)
- DNASTAR package software
- Roter-Gene 3000 (vertion 6.0; Corbett Research)
- Simmonic2005 (version 1.75)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

BIOGRAPHY

NAME: Thaweesak Chieochansin

SEX: Male

DATE OF BIRTH: June 15th, 1982

PLACE OF BIRTH: Bangkok, Thailand

NATIONALITY: Thai

E-MAIL ADDRESS: thch2525@hotmail.com

EDUCATION

2000-2004: Bachelor of Science (2nd Class Honors) in Genetics, Chulalongkorn University, Thailand

2004-present: Ph.D. student, Doctor of Philosophy Program in Biomedical Sciences, Graduate School, Chulalongkorn University, Thailand

RESEARCH GEANT: Royal Golden Jubilee Ph.D. program. Thailand Research Fund

PROFESSIONAL EXPERIENCE: A visiting researcher in Center for Infectious Diseases, The University of Edinburgh, Summerhall, Edinburgh, UK (2008-2009)

PUBLICATION (2004-2009): 5 international publications from this thesis and 15 international publications during study.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย